# Variaciones en el “fitness” del VIH-1 durante la Terapia Antirretroviral

Julia García Prado

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Memoria de tesis para obtener el grado de Doctora por la Universidad Autónoma de Barcelona

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HACEN CONSTAR:

Que el trabajo experimental y la redacción de la tesis titulada: "***Variaciones en el Fitness del VIH-1 durante la Terapia Antirretroviral***" ha sido realizada por Julia García Prado y consideran que es apta para su lectura y defensa ante un tribunal de tesis, y con ello, optar al grado de Doctora por la Universidad Autónoma de Barcelona.

Para que haga constancia en este documento en Badalona, 6 de Junio del 2005

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HACE CONSTAR:

Que el trabajo experimental y la redacción de la tesis titulada: "*Variaciones en el Fitness del VIH-1 durante la Terapia Antirretroviral*" ha sido realizada por Julia García Prado bajo su tutoría y considera que es apta para su lectura y defensa ante un tribunal de tesis y, con ello, optar al grado de Doctora por la Universidad Autónoma de Barcelona.

Para que haga constancia en este documento en Bellaterra, 2005

*"Con un corazón lleno de fantasías delirantes; de las cuales yo soy el capitán;*
*con una lanza de fuego, y en un caballo de viento viajo a través de la inmensidad".*

(*Canción de Tom O´Bedlam.*)

# *Prólogo*

El Virus de Inmunodeficiencia Humana Tipo-1 (VIH-1) es el agente etiológico responsable del Síndrome de Inmunodeficiencia Adquirida (SIDA). Esta enfermedad se caracteriza por una pérdida sostenida de linfocitos T CD4+, provocando una alteración global del sistema inmunitario, y dejando al individuo expuesto a infecciones oportunistas y al desarrollo de ciertas neoplasias.

Desde la introducción de la zidovudina en el año 1986 hasta la implantación a partir del año 1996 de las Terapias Antirretrovirales de Gran Actividad o TARGA (combinaciones de al menos tres compuestos antirretrovirales) se han aprobado un total de 19 nuevos fármacos para el tratamiento de la infección por el VIH-1. En su conjunto, la quimioterapia contra la infección por el VIH-1 incrementa la esperanza de vida de los individuos infectados. Sin embargo, complicaciones surgidas por el uso de estas terapias (p.e una adherencia subóptima, alteraciones en el perfil fármaco-cinético de algunos pacientes o la falta de potencia de los tratamientos) propician la selección de mutaciones de resistencia frente a los fármacos antirretrovirales. La aparición de estas mutaciones en el genoma del VIH-1 constituye una barrera al éxito duradero de las terapias antirretrovirales.

Nuestras investigaciones parten del interés por el estudio de la variación en la eficacia biológica o "fitness" del VIH-1 debido a la adquisición de mutaciones de resistencia. La relación entre el mantenimiento de cepas virales con bajo "fitness" y elevada resistencia con un posible beneficio clínico constituye uno de los ejes centrales en los estudios aplicados de "fitness" viral. A lo largo de este trabajo resumiré el conocimiento acumulado en los últimos años en el estudio del VIH-1: su estructura básica y aspectos más relacionados con esta tesis, como son los fenómenos de resistencia a antirretrovirales y sus consecuencias en la modulación del "fitness" del VIH-1.

# ÍNDICE

## LISTA DE ABREVIATURAS MÁS FRECUENTES

**AZT** 3´azido-3´deoxicitidina, zidovudina

**DNA** Ácido Desoxirribonucléico

**Gp120** Glicoproteína 120

**Gp41** Glicoproteína 41

**GFP** Proteína verde fluorescente

**HTLV-1** Virus Linfotrópico T Humano Tipo-1

**IN** Integrasa

**IPs** Inhibidores de Proteasas

**MLV** Virus de la Leucemia Murina

**p.e** por ejemplo

**PR** Proteasa

**RNA** Ácido Ribonucléico

**SCID/hu** Ratones con Inmunodeficencia Severa Combinada

**SIDA** Síndrome de Inmunodeficiencia Adquirida

**TARGA** Terapia Antirretroviral de Gran Actividad

**TI** Transcriptasa Inversa

**VIS** Virus de Inmunodeficiencia en Simios

**VIH-1** Virus de Inmunodeficiencia Humana Tipo-1

**VSV** Virus de la Estomatitis Vesicular

# CAPÍTULO I

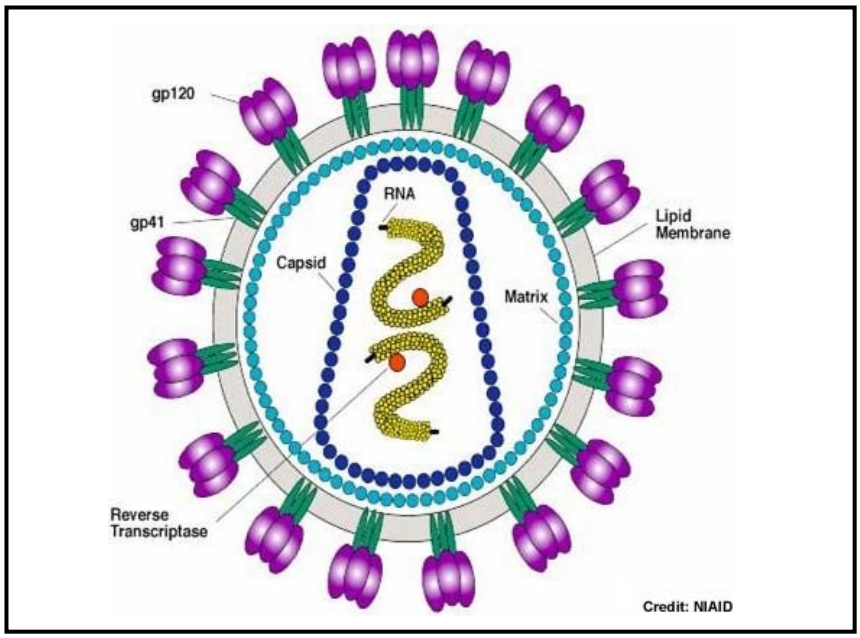
Introducción General

# 1.EL VIRUS

## 1.1 Clasificación y Estructura

El Virus de Inmunodeficiencia Humana tipo-1 (VIH-1) pertenece a la familia *Retroviridae*, un grupo de virus de RNA que replican vía un intermediario de DNA usando una Transcriptasa Inversa. Dentro de los retrovirus el VIH-1 pertenece a la subfamilia *Lentiviridae,* donde se agrupan otros virus relacionados, como son los Virus de Inmunodeficiencia en Simios (VIS).

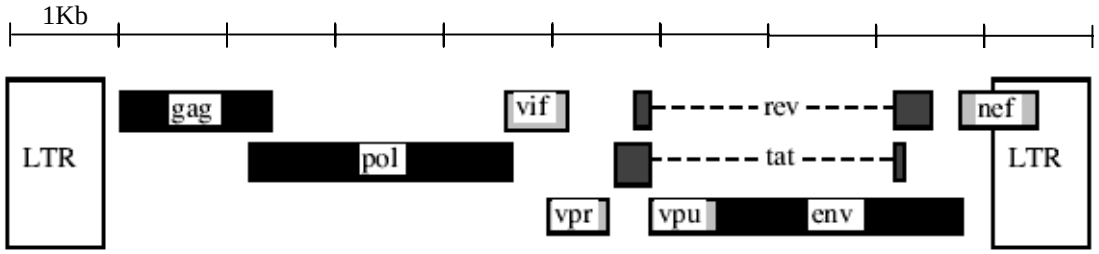
Morfológicamente el VIH-1 está formado por una partícula esférica de aproximadamente 145 nm (Fig.1) en la que se pueden diferenciar varias partes: (i) una interna que contiene dos moléculas de RNA, la nucleoproteína y algunas enzimas virales, la Transcriptasa Inversa, la Proteasa y la Integrasa [TI, PR, IN]; (ii) una intermedia, la cápsida, constituida por un núcleo icosaédrico y (iii) una más externa que forma la envoltura. La envoltura está compuesta por una membrana lipídica derivada de la célula huésped y dos glicoproteínas del virus, gp120 y gp41, imprescindibles para la entrada del virus en la célula [1].




**(Fig.1) Estructura del VIH-1.**

El genoma del VIH-1 (Fig.2) es un RNA de cadena única constituido por 2 hebras idénticas de 9,8 kb y de polaridad positiva. Los 9 genes que componen el genoma del VIH-1 están encargados de codificar componentes de la partícula vírica (genes estructurales) y de regular la expresión de los mismos (genes reguladores).

Los genes estructurales codifican para: (i) proteínas estructurales (*gag* : p17, p24, p2, p7, p1, p6)*;* (ii) enzimáticas (*pol* : TI, PR e IN) y (iii) glicoproteínas de membrana del virión (*env* : gp120 y gp41). Los genes reguladores codifican para: (i) proteínas reguladoras transcripcionales (*Tat*); (ii) post-transcripicionales (*Rev*) y (iii) otras proteínas accesorias (*Vif, Vpu, Nef, Vpr*) que tienen su papel en diversas funciones reguladoras. Asimismo, el VIH-1 codifica secuencias específicas que funcionan en eventos transcripcionales, post-trascripcionales y de ensamblaje [2].




Luciw, P. Capítulo 60, Fields Virology, 3ª Edición

**(Fig.2) Representación esquemática del genoma del VIH-1.**

## 1.2 Ciclo de replicación

El ciclo de replicación de este retrovirus es muy complejo y se puede dividir en varias fases (Fig.3):

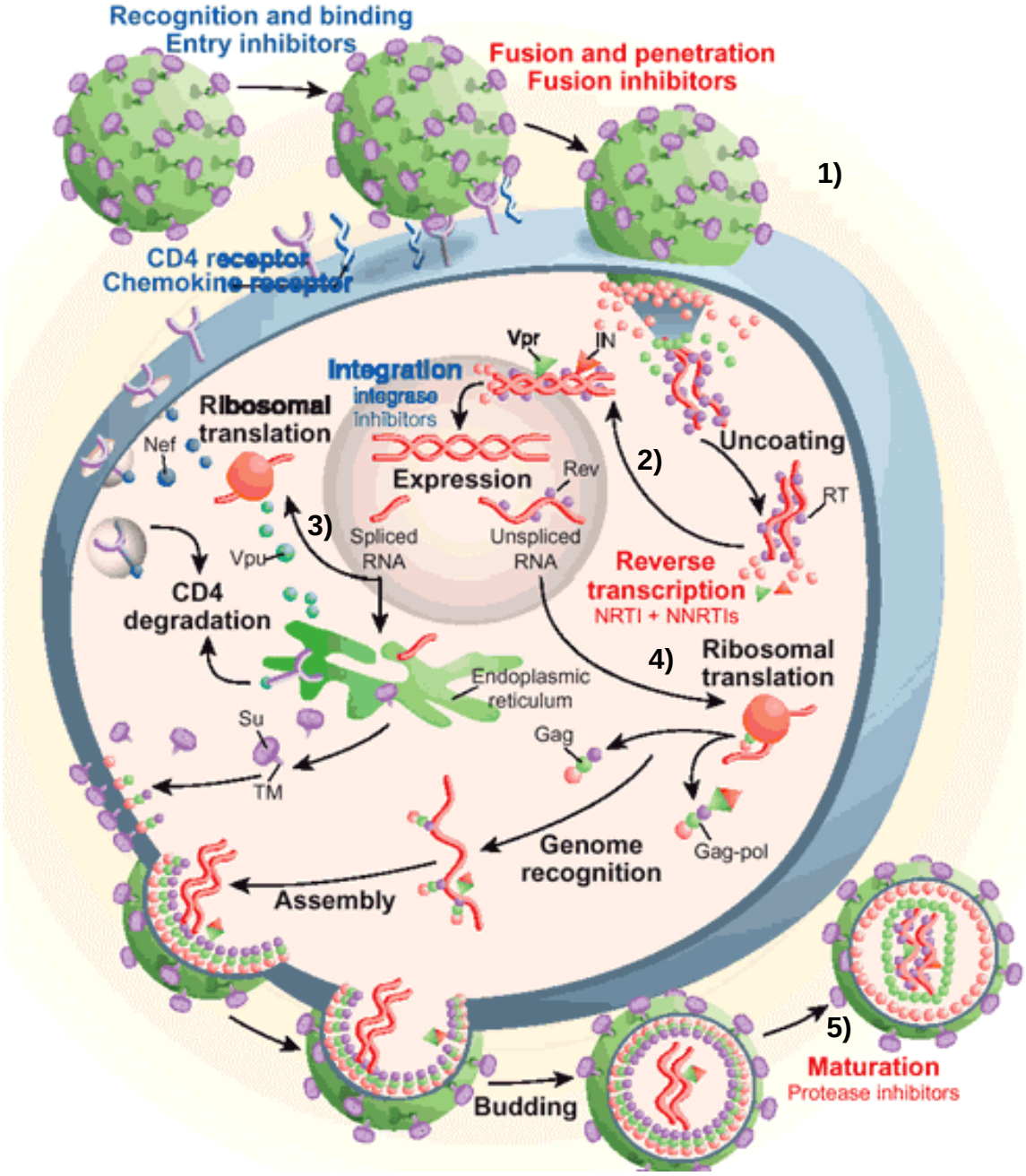
1) **adsorción, fusión e internalización** del virión en el citoplasma celular. Esta parte del ciclo tiene lugar mediante la interacción de las proteínas de la envoltura (gp120 y gp41) con el receptor celular CD4 y el correceptor de la célula T, principalmente CCR5 o CXCR4, permitiendo la entrada del virus en la célula huésped.

2) **transcripción inversa** del RNA viral a DNA por la Transcriptasa Inversa, translocación al núcleo celular **e integración** del genoma viral en la célula por medio de la Integrasa.

3) **expresión temprana de genes** reguladores *(Tat, Vpu, Nef)* durante la transcripción de genes celulares.

4) **expresión tardía de genes** estructurales en forma de precursores poliproteícos *(gag, gag-pol y env).*

5) **salida del virión y maduración proteica** completa de *gag* y *gag-pol* para dar lugar a las diferentes proteínas estructurales: proteínas de la matriz, la cápside o la nucleocápside, proceso llevado a cabo por la proteasa dando lugar a viriones con completa capacidad infectiva.






**(Fig.3) Ciclo de replicación del VIH-1.** En rojo figuran los inhibidores del ciclo de replicación viral. En azul están indicados los inhibidores del ciclo de replicación en fases de desarrollo clínico o preclínico.

# 2. UNA VENTAJA ADAPTATIVA: LA VARIABILIDAD

La variación genética es una característica inherente a todos los virus de RNA y el caso del VIH-1 no es una excepción. La gran heterogeneidad del VIH-1 viene determinada principalmente por varios factores virales (elevado tamaño poblacional, elevada tasa de mutación, dinámica de replicación viral muy activa y procesos de recombinación) y por factores celulares intrínsecos (Tabla.1). El conjunto de estos fenómenos conlleva una acumulación de multitud de pequeños cambios en el genoma y la formación de una estructura poblacional en cuasiespecies. Las **cuasiespecies** se pueden definir como distribuciones complejas de genomas diferentes pero genéticamente relacionadas [3-5]. La presión de selección (pe. los fármacos antirretrovirales o el sistema inmunitario) actúa sobre la compleja estructura poblacional del VIH-1 y no sobre genomas individualizados. Estas presiones favorecen una rápida adaptación del virus a los cambios del medio como consecuencia de la preexistencia, entre el espectro de cuasiespecies, de variantes fenotípicas potencialmente útiles para hacer frente a estos cambios.

**(Tabla.1) Dinámica viral y generación de mutantes resistentes.**

| | |
|---|---|
| Producción diaria de virus | ~ $10^9$-$10^{10}$ virus |
| Producción de virus por célula infectada | ~ $10^2$ virus/célula |
| Ciclos de replicación diarios | ~ $10^7$ ciclos |
| Tasa de error de la TI | ~ 2,5X$10^{-5}$ /sitio/generación |
| Genoma del VIH | ~ $10^4$ nucleótidos |
| Probabilidad de obtener una mutación puntual específica en un codón en particular (cada nucleótido puede mutar a cualquiera de los otros tres nucleótidos) | 0,33 X $10^{-5}$ /ciclo de replicación |
| Producción diaria de virus con una determinada mutación | ~ 3300 virus |
| Producción de mutaciones por genoma en un ciclo de replicación | ~ 0,1-1 mutaciones/genoma/ciclo de replicación |

## 2.1 Tamaño poblacional

Una vez el VIH-1 establece una infección productiva es capaz de mantener una alta tasa de replicación. La elevada replicación da lugar a una producción diaria de virus en torno a $10^9$ –$10^{10}$ partículas virales en pacientes infectados [6]. Este gran tamaño poblacional facilita el aumento de la variabilidad unido al resto de factores.

## 2.2 Tasa de mutación

La TI es un enzima multifuncional, posee actividad DNA polimerasa dependiente de RNA o DNA, actividad endonucleasa H y es responsable de la replicación del genoma del VIH-1. La elevada tasa de mutación del VIH-1 (~$2{,}5\times10^{-5}$ pares de bases por ciclo de replicación) al igual que ocurre con otros virus de RNA (p.e Polio $1{,}3\times10^{-5}$, Virus de la Estomatitis Vesicular $2{,}5\times10^{-5}$) [7], es consecuencia de la falta de actividad exonucleasa 3´-5´ de la TI. La ausencia de la actividad correctora incrementa la probabilidad de incorporación equívoca de nucleótidos cuando se compara con DNA polimerasas celulares, que poseen mecanismos de corrección y reparación.

## 2.3 Tasa de generación

La dinámica poblacional del VIH-1 y su ritmo de recambio se ha estimado en diferentes estudios *in vivo* [8-10]. Según estos cálculos, la vida media de un linfocito activado es de 2 días y la de un virión en plasma de unas 6 horas, dando como resultado que aproximadamente de forma diaria la población viral del plasma se renueva casi en su totalidad.

## 2.4 Recombinación

La capacidad recombinogénica del VIH-1 es de un orden de magnitud mayor que la de otros retrovirus, incluyendo el Virus de la Leucemia Murina (MLV) y el Virus Linfotrópico T Humano tipo-1 (HTLV-1) [11]. Este mecanismo, complementario a todos los anteriores, tiene la potencialidad de acelerar la diversificación y la evolución del VIH-1. La frecuencia de recombinación del VIH-1 varía desde un 9,1 eventos de recombinación por ciclo de replicación en células mononucleadas de sangre periférica hasta 28,7 en macrófagos [12]. El fenómeno de recombinación depende de múltiples factores

[13, 14]. Los procesos de recombinación dan lugar a la formación de híbridos virales, que acumularían mutaciones procedentes de varios genomas, favoreciendo aún más el incremento de la variabilidad.

## 2.5 Hipermutagénesis

Estudios recientes han descrito la presencia de factores celulares que actúan como inhibidores endógenos del VIH-1, uno de ello es el APOBEC3G. Este enzima, miembro de la superfamilia de las citidinas deaminasas celulares, es el causante de los fenómenos de hipermutagénesis (G → A) características de los lentivirus de primates [15-17]. La acción del APOBEC3G tiene como consecuencia la hipermutagénesis letal del RNA viral inhibiendo la replicación por modificación directa del genoma viral o fijando la presencia de nuevas mutaciones (G → A). La proteína viral *Vif* contrarresta la acción del APOBEC3G interaccionando y evitando su incorporación a los viriones y con ello, la activación de los mecanismos de defensa intrínseca mediados por la deaminación del DNA.

# 3. LA APARICIÓN DE RESISTENCIA

Una cuestión que nos surge tras presentar los factores responsables de la variabilidad del virus, son las implicaciones e impacto en la efectividad de las terapias antirretrovirales.

La respuesta requiere tener presente el concepto de cuasiespecies citado en la sección anterior. Los fármacos antiretrovirales, en condiciones subóptimas, favorecen la selección de variantes virales resistentes con mutaciones en los genes diana del fármaco, lo que permite mantener la replicación del virus en las nuevas condiciones ambientales [18-20]. La selección de variantes resistentes como población mayoritaria provoca, de este modo, la falta de efectividad de los fármacos limitando el éxito completo de las terapias antirretrovirales [21-23].

Desde la introducción de la quimioterapia contra el VIH-1 a mediados de los años 80 hasta la actualidad, el diseño y desarrollo de nuevos compuestos ha sido constante llegando a un total de 24 fármacos, formados por 19 principios activos (Tabla.2) [24]. Paralelamente al descubrimiento de estos nuevos compuestos, las terapias antirretrovirales han evolucionado desde monoterapias a Terapias Antirretrovirales de Gran Actividad (TARGA). Estas terapias incluyen tres o más fármacos activos y contribuyen a frenar la tasa de mortalidad por el VIH-1 [25, 26].

Los fármacos antirretrovirales pueden dividirse en tres familias: (i) los **Inhibidores de la Transcriptasa Inversa** con dos subfamilias; los Análogos a Nucleósidos y los No-Análogos a Nucleósidos; (ii) los **Inhibidores de Proteasa** (IPs) y los (iii) **Inhibidores de Fusión**, incorporados recientemente a las terapias antirretrovirales. La parte del ciclo viral sobre el que actúa cada uno de ellos aparece representada en la Fig.3.

Simultáneamente, las mutaciones de resistencia también pueden clasificarse atendiendo al tipo de fármaco antirretroviral que las han seleccionado. En la introducción nos vamos a centrar en la resistencia frente a las dos

primeras familias de fármacos: los Inhibidores de la TI y los Inhibidores de la PR de los que hablaremos en profundidad a lo largo de la tesis.

**(Tabla.2) Fármacos contra el VIH-1.**

## Inhibidores de la Transcriptasa Inversa

### Análogos a Nucleósido

Abacavir (ABC, Ziagen®)

Didanosina (ddI, Videx®)

Emtricitabina (FTC, Emtriva®)

Lamivudina (3TC, Epivir®)

Estavudina (d4T, Zerit®)

Tenofovir[1] (TDF, Viread®)

Zalcitabina (ddC, Hivid®)

Zidovudina (AZT, Retrovir®)

**combinaciones**

Lamivudina+ Zidovudina (Combivir®)

Abacavir+ Lamivudina (Epzicom®)

Tenofovir[1]+ Emtricitabina (Truvada®)

Abacavir+ Lamivudina+ Zidovudina (Trizivir®)

### No-Análogos a Nucleósido

Delavirdina[2] (DLV, Rescriptor®)

Efavirenz (EFV, Sustiva®)

Nevirapina (NVP, Viramune®)

## Inhibidores de proteasa

Amprenavir (APV, Agenerase®)

Atazanavir (ATZ, Reyataz®)

Fosamprenavir (FPV, Lexiva®)

Indinavir (IDV, Crixivan®)

Nelfinavir (NFV, Viracep®)

Ritonavir (RTV, Norvir®)

Saquinavir (SQV, Invirase®)

Lopinavir+ Ritonavir [3](KLT, Kaletra®)

## Inhibidores de fusión

Enfuvirtide (T-20, Fuzeón®)

1.Análogo a nucleótido
2.No aprobado en España para su uso terapéutico
3.Conformación en la que el RTV se añade en dosis reducidas para incrementar los niveles plasmáticos del Lopinavir al inhibir al citocromo P450

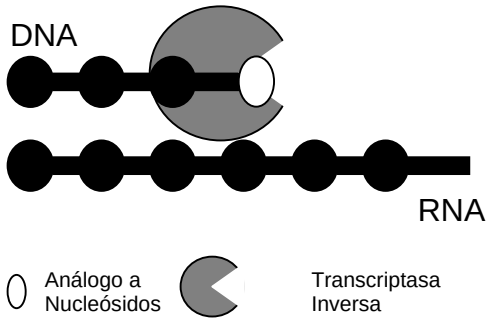
Modificado de Buckheit y cols. 2004

## 3.1 Resistencia a Inhibidores de la Transcriptasa Inversa

La Transcriptasa Inversa es un enzima hetererodimérico codificado por el gen *pol*. Su función principal es la retrotranscripción del RNA viral a DNA anterior a la integración del material genético del virus en el núcleo celular. Los inhibidores de la TI bloquean la elongación de la cadena de DNA naciente. Estos inhibidores se pueden dividir en Análogos a Nucleósido y No-Análogos a Nucleósidos.

### 3.1.1 Análogos a Nucleósidos

Los Análogos a Nucleósidos actúan compitiendo con el sustrato natural del enzima, los deoxinucleósidos. La incorporación de un análogo a nucleósidos al centro activo de la TI bloquea la entrada de nuevos deoxinucleósidos y la elongación de la cadena de DNA naciente.




Modificado de Clavel y cols. 2004

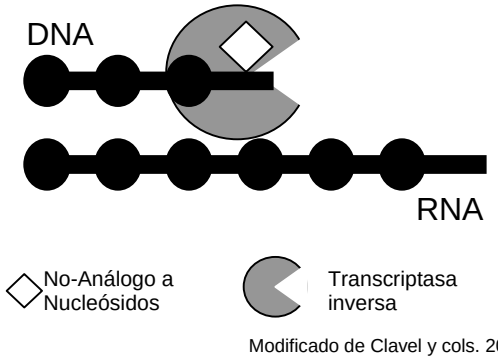
**(Fig.4) Terminación de las síntesis de DNA viral por un Análogo a Nucleósidos.**

Las mutaciones que confieren resistencia a análogos a nucleósidos pueden actuar a través de dos mecanismos: (1) Interfiriendo con la capacidad de la TI para incorporar derivados fosforilados de los correspondientes fármacos. Un ejemplo, es la resistencia al 3TC mediada por la selección de la mutación M184V en la TI. (2) Aumentando la actividad fosforolítica dependiente de ATP o pirofosfato (PPi), lo que permite a la TI eliminar del extremo 3´terminal del DNA aquellos inhibidores que una vez incorporados bloquean la síntesis [27, 28]. Un ejemplo, de este segundo mecanismo es la resistencia a los análogos a timidina mediada por las mutaciones en posiciones 41, 67, 70, 210, 215 y 219.

Cada uno de estos dos mecanismos están caracterizados por ciertas mutaciones de resistencia (ver Tabla 3). Las mutaciones de resistencia a los análogos a nucleósidos se localizan principalmente entorno al centro activo del enzima, aunque altos niveles de resistencia a estos fármacos sólo se alcanzan tras la acumulación de múltiple mutaciones.

### 3.1.2 No-Análogos a Nucleósidos

El caso de los No-Análogos a Nucleósidos es diferente. Estos inhibidores no competitivos interaccionan en un dominio hidrofóbico próximo al centro activo de la TI. Los no-análogos actúan como inhibidores estéricos dificultando la movilidad de la TI y bloqueando la polimerización.




Modificado de Clavel y cols. 2004

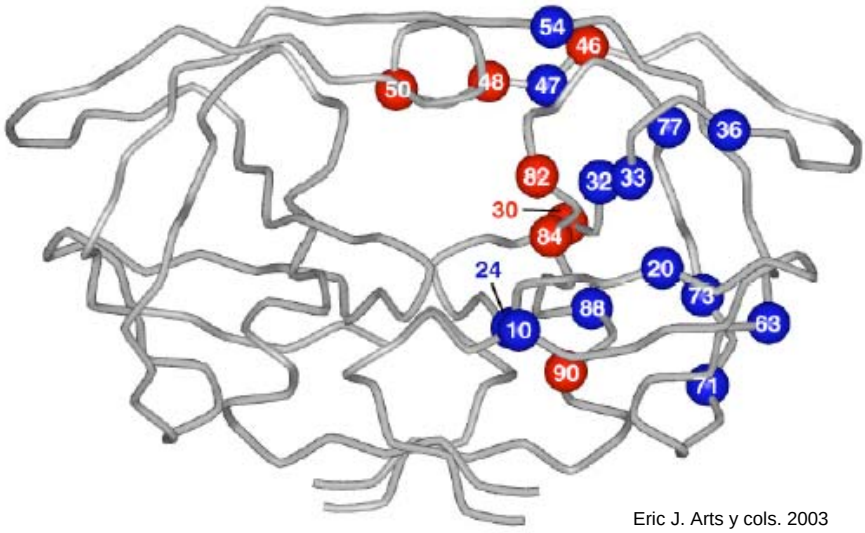
**(Fig.5) Terminación de las síntesis de DNA viral por un No-Análogo a Nucleósidos.**

El mecanismo de resistencia de los no-análogos está asociado a la naturaleza de las fuerzas con su sitio de unión. La alteración de las débiles interacciones químicas entre el enzima y el no-análogo, debida a cambios aminoacídicos, son suficientes para conferir resistencia. Las mutaciones de resistencia durante una terapia con no-análogos se seleccionan de forma muy rápida. Estas mutaciones se posicionan cercanas al sitio de unión de los no-análogos y, al contrario de los análogos, mutaciones puntuales confieren un grado elevado de resistencia (ver Tabla 3).

## 3.2 Resistencia a los Inhibidores de Proteasa (IPs)

Los IPs actúan de forma específica bloqueando el procesamiento por parte de la PR viral de moléculas polipeptídicas (*gag* y *gag-pol*). El procesamiento de *gag* y *gag-pol* es necesario para la maduración completa del virus y la formación de partículas infecciosas. Los IPs son inhibidores que compiten con el sustrato natural de la PR por la unión a su centro activo.

A pesar del pequeño tamaño de su estructura homodimérica, sólo 99 aminoácidos, la PR del VIH-1 es capaz de acumular un gran número de mutaciones de resistencia manteniendo su actividad [29]. Se han descrito hasta 49 polimorfismos naturales y 20 mutaciones asociadas a resistencia a IPs (Fig.6). Estas mutaciones se pueden localizar en el centro activo del enzima (**mutaciones primarias**) confiriendo un elevado grado de resistencia o en otras regiones de la PR modulando su actividad, pero sin interferir directamente con la unión de los IPs al sustrato (**mutaciones secundarias**).




**(Fig.6) Estructura tridimensional de la proteasa del VIH-1**. Las esferas de colores representan mutaciones de resistencia; en rojo mutaciones primarias y en azul mutaciones secundarias.

La acumulación de varias mutaciones es necesaria para conseguir un nivel alto de resistencia a la mayor parte de los IPs por lo que se dice que son fármacos con elevada barrera genética. Además, su similitud estructural hace que algunas mutaciones confieran niveles altos de resistencia a varios IPs al mismo tiempo, dando lugar a fenómenos de **resistencia cruzada**.

No sólo se han descrito mutaciones de resistencia a IPs localizadas en la PR, sinó también, se han descrito diferentes sustituciones aminoacídicas en los sustratos naturales de la PR [30-32]. Estas mutaciones se localizan cerca de los **sitios de procesamiento** de la poliproteína gag (p2/p7, p7/p1 y p1/p6) por la PR. Se ha visto que algunas de estas mutaciones tienen un papel importante en **coevolución** con la PR [33]. El significado biológico de estas mutaciones es contradictorio y está aún por esclarecer [34]. Algunas mutaciones se han asociado con un aumento del nivel de resistencia y de capacidad replicativa del virus, facilitando el procesamiento proteico o mejorando la habilidad de las proteasa para interaccionar con el sustrato [35-38]. Una discusión más amplia acerca del papel de estas mutaciones en los fenómenos de resistencia y su posible efecto modulador de la capacidad replicativa aparece en capítulos posteriores.

**(Tabla.3) Mutaciones más comunes en aislados clínicos, implicadas en resistencia del VIH-1 a inhibidores de la transcriptasa inversa Análogos a Nucleósidos, No Análogos a Nucleósidos e IPs.**

| **Mutaciones Transcriptasa inversa** | **Comentarios** |
|---|---|
| **Mutaciones que confieren resistencia a los análogos a nucleósidos** | Familia de mutaciones conocida como mutaciones a análogos a timidina. Asociada con resistencia a la mayor parte de análogos a nucleósidos excepto a la lamivudina. |
| M41L<br>D67N<br>K70R | In Vitro, causan un elevado nivel de resistencia a zidovudina y bajo nivel de resistencia a estavudina, didanosina y abacavir. |
| L210W<br>T215Y, T215F<br>K219Q, K219E | Segregada en dos rutas, una en la que está implicada T215Y y L210W y la otra T215F y K219Q. Ruta en la que están implicadas también la T215Y y la L210W y asociada con una baja respuesta al tenofovir. |
| M184V | Presente en la mayor parte de los virus resistentes a la lamivudina. Confiere una elevada resistencia a la lamivudina in vitro. Puede interferir en la resistencia a zidovudina y estavudina cuando el número de mutaciones a análogos de timidina es muy bajo. |
| A62V<br>V75I<br>F77L<br>F116Y<br>Q151M | Ruta poco usual de resistencia del VIH-1 a los análogos a nucleósido. In Vitro, causa un elevado nivel de resistencia a la mayor parte de los análogos a nucleósidos excepto a la lamivudina y al tenofovir. |
| Inserción 69 | Inserción de 2 o más aminoácidos (usualmente serinas) a lado del codón 69. Emerge sólo en virus que previamente tienen varias mutaciones a análogos a timidina. Confiere un elevado grado de resistencia a todos los análogos a nucleósidos. |
| K65R | Seleccionada en terapias con zalcitabina, abacavir y tenofovir. |
| L74V | Seleccionada en terapias con didanosina, ususalmente cuando la didanosina es el único análogo Seleccionada en terapias con abacavir. |
| Y115F | |
| **Mutaciones que confieren resistencia a los No-análogos a nucleosidos** | |
| K103N | Mutación normalmente seleccionada en terapias con efavirenz. Ocasionalmente seleccionada en terapias con nevirapina. Confiere una elevada resistencia a todos los no-análogos a nucleósidos. |
| V108I<br>Y181C<br>Y188C | Mutaciones seleccionadas en presencia de nevirapina. Confiere un alto nivel de resistencia a nevirapina pero bajo a efavirenz. Y188L, a diferencia de la Y188C, se observa principalmente en terapias con efavirenz. |
| L100I<br>V106A<br>G190A,G190S | Mutaciones que se acumulan durante terapia prolongada inefectiva a la mayor parte de los no análogos a nucleósidos. |
| **Mutaciones en la proteasa y gag** | |
| L24I | Aparece durante el fracaso a terapias con indinavir y en terapias con lopinavir. |
| L47V,I50V | Seleccionadas en terapias con amprenavir y también lopinavir. |
| G48V | Se selecciona de modo exclusivo bajo terapia con saquinavir, asociada a un elevado nivel de resistencia a saquinavir. |
| I84V | Se encuentra con frecuencia después de una terapia prolongada inefectiva a IPs. Asociada a altos niveles de resistencia a la mayor parte de los IPs |
| L90M | Mutación de resistencia frecuente, se observa durante el fracaso de la terapia a la mayor parte de los inhibidores de proteasa. Normalmente se selecciona en presencia de saquinavir. |
| V82A, V82T, V82F | Mutaciones comunes de resistencia, pueden emergen de modo temprano al tratamiento con la mayor parte de los inhibidores de proteasa. Se seleccionan principalmente en presencia de ritonavir e indinavir. |
| D30N<br>N88D, N88S | Mutaciones seleccionadas en presencia de nelfinavir. D30N aparece siempre primero. |
| L10I, L10F<br>K20R, K20M<br>M36I,M46I, M46L<br>I54V, I54L<br>A71V, A71T<br>G73S, V77I, M93L | Mutaciones que se pueden acumular durante el fracaso a una terapia con inhibidores de proteasas causando un incremento gradual en el nivel de resistencia. |
| A431V, L449F | Mutaciones en gag, el principal sustrato de la proteasa, aumentan la resistencia y compensan parcialmente la pérdida de capacidad replicativa asociada a resistencia. |
| | Modificado de Clavel y cols. 2004 |

El desarrollo de mutaciones de resistencia es la respuesta del virus a las nuevas condiciones ambientales moduladas por la presencia de los fármacos antirretrovirales. Pero, ¿cuál es el coste biológico que le supone al VIH-1 el mantenimiento de estas mutaciones de resistencia?, ¿qué es el "fitness viral"?, ¿por qué se modifica y cómo se puede cuantificar?, ¿tiene algún significado clínico?. Estas preguntas son las que trataremos de responder a lo largo del resto de la introducción constituyendo la base de este trabajo de tesis.

## 4. "FITNESS" VIRAL

Cada vez que se cita la palabra "fitness" se pone de relieve la confusión y diferencia de opiniones sobre su significado. Dado que el estudio del "fitness" es la intersección entre genética de poblaciones, virología y medicina clínica, es sorprendente que no haya incluso mayor confusión [24, 39-42].

El **"fitness"** se puede definir, de un modo más formal, como la capacidad para transmitir con éxito un material genético concreto a la siguiente generación [39]. Otra definición podría ser la capacidad de producir una progenie infecciosa estable en un determinado ambiente [23]. Desde un punto vista purista el "fitness" sería un parámetro dinámico que englobaría la totalidad de factores virales y del huésped asociados a una eficacia biológica. Sin embargo, el uso de la palabra "fitness" junto con capacidad replicativa o infectividad suelen llevar a confusión. Capacidad replicativa e infectividad se reducirían a aquellos valores obtenidos de modo experimental, limitados al estudio de virus concretos en ausencia de otros factores ambientales. En la actualidad el uso de estas palabras es muy diferente, habiéndose entremezclado los tres términos a lo largo de estos años hasta hacerse indistinguibles en la mayor parte de publicaciones. De esta observación parte nuestra decisión de usar el término "fitness" a lo largo de la tesis.

El "fitness" no es un fenómeno exclusivo de los virus de RNA (VIH-1, FMDV, VSV, entre otros) [3, 43]. También es aplicable a otros microorganismos como bacterias [44] o levaduras [45]. En los virus de RNA y en el caso del VIH-

1 la modulación del "fitness" viral tiene un componente multifactorial. La presión ejercida por los fármacos antirretrovirales durante las terapias y la presión ejercida por el sistema inmunitario actúan como componentes moduladores [46]. Las presiones de selección suponen una desventaja para aquellas variantes virales con elevada susceptibilidad a los fármacos o, aquellas sobre las cuales la respuesta inmunitaria es muy eficaz. Estas presiones favorecen la selección de variantes resistentes a los fármacos o que han encontrado un mecanismo de escape inmunitario [47].

El coste biológico, que supone en algunas ocasiones, para el virus la aparición de estas variantes resistentes, así como, el posible beneficio clínico del establecimiento de cepas virales con baja "fitness" (poca capacidad replicativa), ha despertado en los últimos años un gran interés por el estudio del "fitness" viral en el VIH-1. El estudio de estos fenómenos de "fitness" serán ampliamente discutidos a lo largo de toda la tesis.

## 4.1 ¿Cómo se estima el "fitness"?

La teoría del estudio del "fitness" parte de modelos matemáticos de genética de poblaciones basados en la comparación de dos variantes poblacionales, una salvaje y otra mutante [48-50]. En estos estudios se concluye que la ventaja selectiva es la fuerza crítica que influencia la composición genética de la población. Para el modelaje del "fitness" hay que tener en cuenta toda una serie de factores: virales (tasa de mutación, tamaño de la población que replica, tasa de replicación, etc) y del huésped (tasa de mortalidad de las células infectadas, eliminación del virus, etc), lo que hace del "fitness" una característica no definitoria sino dependiente de otras.

Mientras que la descripción de "fitness", en el ámbito matemático, tiene su base en la genética de poblaciones, la medida de un modo experimental es mucho más compleja. La dificultad de estandarizar un método y la variabilidad de resultados usando diferentes metodologías ha abierto el debate

acerca de cúal es el método de medida más correcto y representativo de lo que ocurre *in vivo* [51].

## 4.2 ¿Es posible medir la contribución de secuencias individuales al "fitness" del virus?

Las medidas de "fitness" viral se efectúan sobre patrones mutacionales concretos asociados a resistencia frente a fármacos antirretrovirales. Estos estudios se realizan mediante técnicas moleculares que permiten introducir mutaciones puntuales o genes concretos en cepas virales isogénicas. Los clones provirales serán idénticos en todas las posiciones excepto en aquellas objeto de estudio. No obstante, las interacciones entre las diferentes partes del genoma para modular el fenotipo final de los virus, denominados procesos de epistasis [52, 53], podrían no estar reflejadas cuando estudiamos un único determinante genético fuera de su contexto original. Esta característica hace que en la medida del "fitness" el todo será diferente de la suma de las partes. Por el contrario, algunos trabajos han hecho uso de aislados virales completos recuperados *ex vivo* a partir de linfocitos estimulados. El trabajo experimental con estos aislados virales completos entorpece la comprensión del efecto de un solo cambio entre el elevado número presente en el genoma viral completo [54, 55]. Estas son algunas de las limitaciones en la búsqueda de modelos experimentales de "fitness", modelos que tendrían que ser rápidos, reproducibles y sobre todo biológicamente relevantes.

## 4.3 Modelos experimentales para estimar "fitness" viral

El abordaje del diseño de modelos experimentales para la medida de "fitness" viral se ha llevado a cabo bajo diversas perspectivas y niveles de complejidad. Las medidas varian desde el estudio *in vitro* de las actividades catalíticas de los enzimas diana de los fármacos antirretrovirales, hasta el uso de modelos animales *in vivo* en ratones o macacos.

A continuación, aparece una lista completa de las diversas metodologías usadas para la medida del "fitness" viral tanto *in vitro* como *in vivo* [39, 40, 51].

### 4.3.1 Modelos experimentales "*in Vitro*"

Estos modelos se realizan en el ámbito bioquímico calculando la actividad catalítica de diferentes enzimas virales o mediante diferentes técnicas de cultivo celular.

- **Ensayos enzimáticos.** El objetivo de estos ensayos es cuantificar la modificación de la actividad catalítica de diferentes enzimas, normalmente PR y TI, debido a la adquisición de mutaciones de resistencia [56-58].
- **Ensayos de un solo ciclo de replicación.** Estos experimentos van destinados a la medida de un modo rápido, 24 a 48 horas, de la capacidad replicativa del virus. Se llevan a cabo con *pseudovirus* o diferentes líneas celulares que contienen un gen marcador (luciferasa, β-galactosidasa, GFP, etc), y permiten cuantificar con facilidad la infección viral [54, 59].
- **Cinéticas de varios ciclos de replicación.** Estos ensayos se suelen realizar en líneas linfoides inmortalizadas (MT2, MT4, CEM, etc) o en cultivos de linfocitos primarios. Experimentalmente obtenemos datos de la tasa de replicación de cada una de las variantes en cultivos aislados a lo largo del tiempo [60, 61].
- **Ensayos de competición entre dos variantes virales**. Los experimentos de competición son los más rigurosos. En estos experimentos se ponen en cocultivo dos virus diferentes y se miden las proporciones relativas de cada una de las variantes virales a lo largo del tiempo de cocultivo. Estos experimentos se repiten en series con diferentes multiplicidades de infección, siendo muy laboriosos y largos, sobre todo si se realizan con aislados clínicos de VIH-1. Están enfocados principalmente a la

evaluación de genes específicos como pueden ser la PR, la TI [62, 63] o a la comparación de diferentes subtipos [64] .

- **Cultivo de timo, nódulo linfático u otros tejidos linfoides** que permitan diseccionar el "fitness" de aislados virales completos. Estos ensayos van destinados a cuantificar la patogenicidad de las variantes virales midiendo la mortalidad de células T CD4 presentes en los cultivos [65, 66].

### 4.3.2 Modelos experimentales "*In Vivo*"

- **Infecciones en animales.** Estos ensayos son mucho más laboriosos y difíciles de estandarizar en comparación a los citados hasta el momento. En estos experimentos se usan simios o ratones. La infección por VIH-1 está restringida en simios, y sólo mediante la formación de quimeras virales (VIH-1/VIS) se logra una infección eficiente en estos animales. El modelo en primate para el estudio de patogénesis del VIH-1, por tanto, está sujeto a la infección por virus cuyo genoma es una mezcla de VIH-1 para los genes que se pretende evaluar y VIS. Otro modelo alternativo es el uso de ratones SCID/hu, ratones con inmunodeficiencia severa combinada, a los cuales se les ha implantado timo, nódulos linfáticos o bazo humano para cuantificar patogénesis y "fitness" [65].

- **Ensayos clínicos**. Una forma de evaluar *in vivo* el "fitness" de diferentes variantes virales se han obtenido de los protocolos clínicos de interrupciones del tratamiento. La velocidad de sustitución de variantes con mutaciones de resistencia por la cepa salvaje durante el periodo de interrupción permite cuantificar, de un modo indirecto, la diferencia de "fitness" entre las poblaciones presentes en cada uno de los momentos. Estos estudios son una conjunción entre datos experimentales y modelos matemáticos de genética de poblaciones [67-70].

## 4.4 “Fitness” y resistencia

Lo descrito hasta el momento sienta la base del estrecho vínculo que existe entre resistencia a antirretrovirales y “fitness” viral. El coste biológico que supone para el virus la adquisición de mutaciones de resistencia se ve reflejado en una modificación del “fitness” viral. No obstante, no todas las mutaciones de resistencia asociadas a fármacos antirretrovirales tienen un impacto similar sobre el “fitness”del VIH-1. Varios trabajos han descrito que las variantes resistentes tienen un “fitness” menor que la cepa salvaje en ausencia de fármaco [71, 72]. Las mutaciones que aparecen primero durante la terapia antirretroviral conferiran un elevado grado de resistencia y coste de “fitness”, mientras que mutaciones acumuladas posteriormente, denominadas mutaciones compensatorias, favorecerían la recuperación del “fitness” en la mayor parte de los casos reportados. Sin embargo, la utilidad clínica y las consecuencias de la disminución de la capacidad replicativa en los virus resistentes no están claras.

El uso prolongado de las terapias antirretrovirales ha favorecido un incremento en el número de pacientes que desarrollan resistencia frente a los fármacos antirretrovirales. El crecimiento constante de métodos para medir resistencia [73] ha apoyado los beneficios clínicos del genotipo y del fenotipo a la hora de tomar decisiones terapéuticas [74]. A pesar de ello, hoy en día la variabilidad genética del VIH-1 y el desarrollo de resistencia excede las opciones terapéuticas, produciendo un aumento en el número de pacientes con experiencia farmacológica y resistencia a todos los fármacos antirretrovirales. Las opciones terapéuticas en estas circunstancias son limitadas. Desde el punto de vista clínico el conocimiento del “fitness” viral podría ser muy útil como parámetro adicional asociado a un genotipo concreto.

## 4.5 “Fitness” y patogénesis

La relación entre “fitness” y patogénesis surgió a partir de varias publicaciones en las que se sugería una menor virulencia de los virus resistentes en comparación con cepas salvajes [75, 76], asociando esta disminución de virulencia a una progresión más lenta de la enfermedad. Aunque varios estudios clínicos han esbozado este fenómeno [77, 78], se ha descrito una menor patogenicidad en timo de variantes resistentes a IPs y también una menor “fitness” de aislados procedentes de pacientes lentos progresores [76]. Ningún trabajo a gran escala ha corroborado de una forma definitiva estas observaciones.

Un obstáculo a la hora de establecer correlaciones entre patogénesis y “fitness” son las diferencias entre las metodologías usadas para la medida de estos factores. Igualmente, hay que tener en cuenta que los factores del huésped juegan un papel importante en la modulación de ambos parámetros y no son evaluables en la mayor parte de estudios realizados *in vitro.*

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# *OBJETIVOS*

Los objetivos planteados a lo largo de la tesis se enumeran a continuación:

*1. Conocer el impacto de determinadas mutaciones de resistencia a fármacos antirretrovirales en el "fitness" del VIH-1.* Con el fin de abordar este objetivo, iniciamos nuestros estudios trabajando con virus resistentes a los IPs (capítulo II) para posteriormente trabajar con virus multirresistentes a los inhibidores de la TI (capítulo III).

*2. Evaluar cambios de "fitness" en pacientes que han pasado por todos los tipos de terapia desde el inicio de la pandemia.* El objetivo de este estudio es observar las variaciones de "fitness" del VIH-1 bajo la influencia de diversos tratamientos antirretrovirales (capítulo IV).

*3. Desarrollar metodologías adecuadas para las medidas del fitness y capacidad replicativa a fin de poder optimizar estos ensayos, y establecer criterios para el uso de cada una de las técnicas.* (capítulo II-IV).

# CAPÍTULO II

## Amprenavir Resistant HIV-1 Exhibits Lopinavir Cross-Resistance and Reduced Replication Capacity

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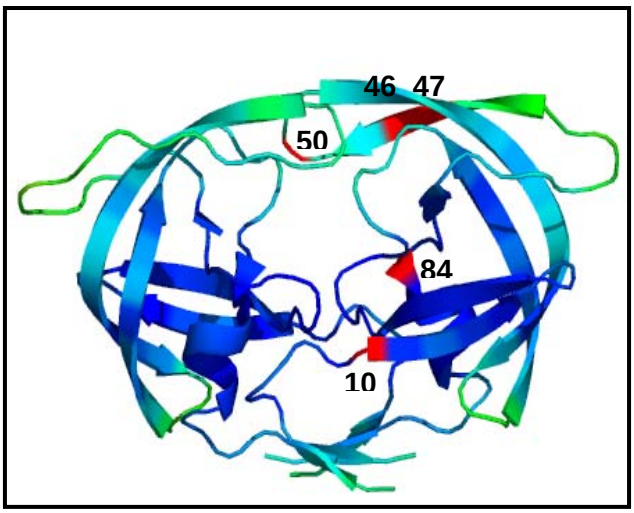
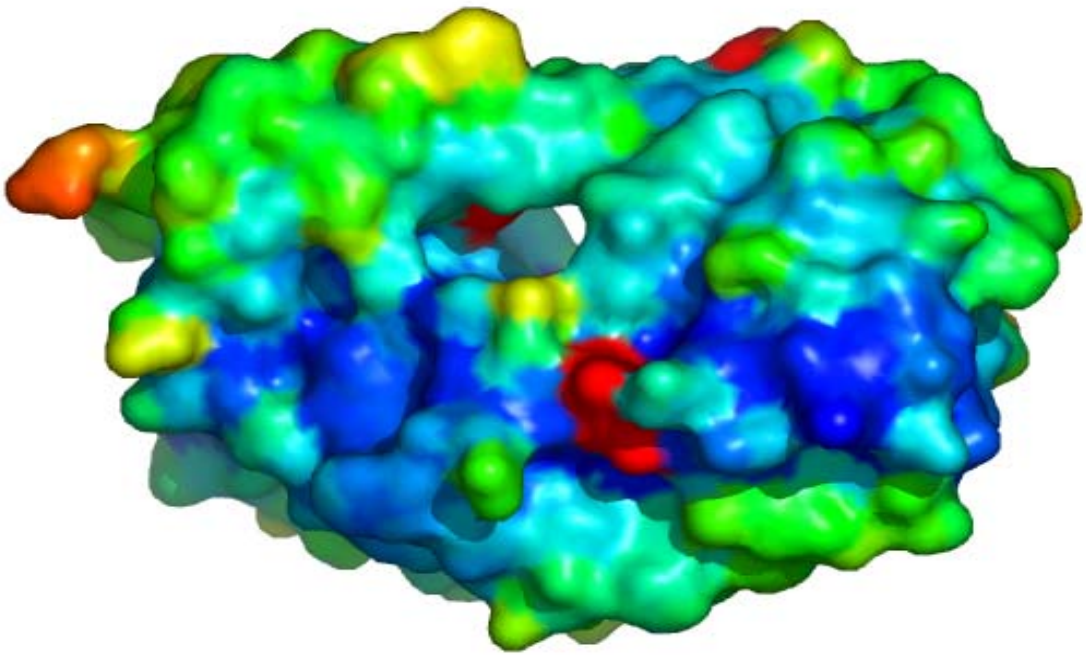


## Proteasa del VIH-1

Estructura de la proteasa cristalizada, resolución 1,90 angstroms; B.Pillai y cols 2001.Modificada en Pymol. En rojo aparecen representadas las posiciones donde se seleccionan mutaciones de resistencia al Amprenavir.

El Amprenavir es un IP introducido a finales de los años 90 en las terapias antirretrovirales y que, junto con el resto de los IPs, ha supuesto un cambio de concepción en el tratamiento antirretroviral. Cabe remarcar que el Amprenavir es también la molécula activa del Fos-amprenavir, compuesto recientemente aprobado para su uso clínico. Este trabajo presentado en este capítulo, surge de la necesidad de estudiar el impacto de las mutaciones de resistencia al Amprenavir en el "fitness" del VIH-1 y la posible resistencia cruzada de este fármaco con otros IPs.

Los virus seleccionados *in vitro,* en concentraciones crecientes de Amprenavir, acumulan progresivamente mutaciones en posiciones 10, 46, 47, 50 and 84 de la proteasa junto con la mutación 449 en Gag. Estos virus nos permiten evaluar, de un modo aislado y progresivo, el impacto de las mutaciones en los fenómenos de resistencias y de "fitness" viral. Las mutaciones de resistencia al Amprenavir disminuyen el "fitness" del VIH-1, y lo hacen de forma progresiva a medida que se acumulan mutaciones y aumenta la resistencia. Además, estas mutaciones de resistencia al Amprenavir provocan resistencia cruzada a otros IPs, sobre todo al Lopinavir y en menor grado al Ritonavir. Estos fenómenos de resistencia cruzada limitarán las opciones terapéuticas en aquellos pacientes que no respondan a un tratamiento que contenga Amprenavir.

## ABSTRACT

***Objectives:*** To evaluate protease inhibitor cross-resistance and reductions in replication capacity conferred by amprenavir-selected mutations.

***Methods:*** HIV-$1_{IIIB}$ variants derived from passage in increasing concentrations of amprenavir were studied, as well as 3'Gag/protease recombinants derived from them. These strains progressively accumulated mutations at codons 10, 46, 47, 50 and 84 in the protease as well as a p1/p6 cleavage site mutation at codon 449 in Gag. Their susceptibility ($IC_{50}$) to various protease inhibitors and their corresponding replication capacities were evaluated by a single-cycle growth assay and compared to measures using competitive cultures and p24 antigen production.

***Results:*** Amprenavir susceptibility decreased with increasing numbers of protease mutations. Changes in lopinavir susceptibility paralleled changes in amprenavir susceptibility. Certain amprenavir-selected mutants conferred >10-fold cross-resistance to lopinavir, including L10F/M46I/I50V-GagL449F (19-fold) and L10F/M46I/I47V/I50V-GagL449F (31-fold). Moreover, one isolate with only 2 mutations in the protease (L10F/84V) and GagL449F displayed a 7.7-fold increase in the lopinavir $IC_{50}$. Low-level cross-resistance to ritonavir and nelfinavir was also observed. The replication capacity of viruses containing either I84V or I50V was at least 90% lower than the reference virus in the single-cycle assay. The order of relative replication capacity was wil-type >L10F >L10F /I84V> L10F/M46I/I50V >L10F/M46I/I47V/I50V.

***Conclusions:*** These results indicate that until more comprehensive genotype-phenotype correlations between amprenavir and lopinavir susceptibility are established, phenotypic testing may be preferable to genotyping for detection of cross-resistance, and should be considered when switching patients from a failing amprenavir-containing regimen. This study also provides data on the concordance of replication capacity measurements generated using a rapid single-cycle growth and competition assays.

## INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) protease inhibitors (PIs) block the processing of the Pr55Gag and Pr160Gag/Pol polyprotein precursors by protease (PR). Highly active antirretroviral therapies (HAART) that contain PIs, have led to dramatic decreases in plasma viremia and corresponding reductions in morbidity and mortality [1]. However, the relatively rapid emergence of viruses that are resistant to these compounds substantially limits long-term treatment efficacy of currently available regimens [2, 3].

In the present study, analysis was performed on HIV-1 variants derived previously by selection in vitro for resistance to amprenavir (formerly VX-478 or 141W94) [4, 5]. Viruses selected in vitro for decreased susceptibility to amprenavir [6] carried PR mutations similar to those observed in patients treated with amprenavir [7]. Isolates from amprenavir-treated patients exhibit decreased amprenavir susceptibility and have mutations in the HIV-1 protease gene resulting in amino acid substitutions, primarily at positions M46I/L, I47V, I50V, I54L/V, and I84V, as well as mutations in the Gag p1/p6 cleavage site [7]. Varying degrees of HIV-1 cross-resistance among protease inhibitors have been reported previously [6, 8]. However, protease inhibitor cross-resistance in HIV-1 isolates from amprenavir-treated patients has not been fully evaluated.

The accumulation of mutations in virus grown in increasing concentrations of amprenavir in vitro [6] provided an opportunity to dissect the interrelated effects of mutations on amprenavir resistance and replication capacity. Cross-resistance to other PIs, including the newest compound available for widespread use (formulated in a fixed combination with ritonavir as Kaletra™), lopinavir, was also studied

## MATERIALS AND METHODS

***Viruses.*** Viruses emerged during serial passages of HIV-$1_{IIIB}$ in the presence of increasing concentrations of amprenavir, as previously reported [6]. The sequence of emergence of mutant virus was L10F (M1c$^+$), L10F/I84V (M2c$^+$), L10F/M46I/I50V (M4c$^+$) and L10F/M46I/I47V/I50V (M5c$^+$). Each of these viruses had a L449F mutation in the Gag p1/p6 site that is cleaved by PR (designated by "+" in the names). Gag-Pol PCR products amplified from these amprenavir-selected $HIV_{IIIB}$ isolates were cloned into a genomic proviral vector (pJM11ΔGPR) [9] derived from $HIV_{NL4-3}$. This produced the following recombinant viruses: L10F/I84V (M2r$^-$), L10F/I84V/Gag L449F (M2r$^+$), L10F/M46I/I50V/Gag L449F (M4r$^+$) and L10F/M46I/I47V/I50V/Gag L449F (M5r$^+$). The "$^-$" indicates lack of the mutation in Gag p1/p6 cleavage site (e.g., the wild-type 449L). To serve as a control, a recombinant virus containing the corresponding Gag-Pol fragment of HIV-$1_{IIIB}$ was cloned into the same vector (IIIB$^-$). A mutant virus containing PR I50V was constructed by site-directed mutagenesis in $HIV_{NL4-3}$ isogenic background [71]. *Pol* and Gag (p7, p1 and p6) sequences in all viral vectors were confirmed before virus production. Stocks of virus were generated in MT-2 cells by electroporation. The 50% tissue culture-infectious dose ($TCID_{50}$) of each virus stock was determined by endpoint dilution in MT-2 cells [11].

***Drug susceptibility.*** A rapid recombinant assay was used to measure the drug susceptibility of the $HIV_{IIIB}$ variants selected in vitro, the recombinant $HIV_{NL4-3}$ virus clones derived from each $HIV_{IIIB}$ isolate, and isogenic viruses derived by site directed mutagenesis (PhenoSense HIV, ViroLogic) [72]. This assay involves the construction of resistance test vectors, which are comprised of a pool of recombinant HIV-1 containing Gag (3'-end from p7), PR and reverse transcriptase (RT) sequences derived from the virus sample that is being evaluated. Resistance test vectors also contain a luciferase reporter gene replacing *env* to monitor a single round of virus replication.

Susceptibility of resistance test vectors to a panel of HIV-1 protease inhibitors was compared to a reference vector containing the PR and RT sequences derived fromHIV-$1_{NL4-3}$. Two independent measurements of each

viral isolate were obtained. The correlation in the $IC_{50}$ between different drugs (measuring the degree of cross-resistance) was estimated using Spearman`s rank correlation test.

***Replication capacity***. Replication capacity was measured using a modified version of the PhenoSense drug-susceptibility assay [13, 14]. The relative replication capacity of the virus was determined by measuring the amount of luciferase activity produced 72 hours after infection in the absence of drug. Replication capacity is expressed as the percentage of the luciferase activity produced by the vectors containing mutant Gag-Pol sequences compared to the luciferase activity from vectors containing the HIV-$1_{NL4-3}$ Gag-Pol reference sequences (100%). Two replicates were performed for each mutant. The replicative capacity of each mutant, measured as a percentage of that of wild-type, was normalized using a square-root transformation. Differences between viral strains in their replication capacity were tested by fitting a mixed effects model using maximum likelihood, which assumed that the replication capacity of each strain was a fixed effect, and between-replicate error was a random effect.

***Replication kinetics.*** Inocula of 3,000 $TCID_{50}$ of each virus were used to infect 3 X $10^6$ MT-2 cells (multiplicity of infection [MOI] of 0.001) as previously described [71]. Cultures were set up in 24-well plates, with 5 replicates per mutant. p24 growth kinetics was analyzed by fitting a linear model to the log-transformed p24 data from the five replicates for each mutant by maximum likelihood. For a given mutant, the growth rate of p24 was assumed to be the same between replicates, but the initial level of p24 was allowed to vary between replicates according to a normal distribution. Missing values were assumed to be missing at random. This model was found to give a good fit to the data.

***Replication competition.*** Growth competition assays were performed in MT-2 cells in the absence of drug as described elsewhere [73]. Infections were initiated with unequal amounts of two competing virus variants; typically 20% and 80% based on virus infectivity titrations. MT-2 cells were also separately infected with mutants in the absence of WT virus to evaluate potential for true genetic reversion over the

course of the experiment. Aliquots containing cells were taken at days 1, 4, 7, 14 and 21, and total infected cell DNA was extracted (Qiamp DNA blood Mini kit, Qiagen). DNA was PCR-amplified in triplicate including the 3´-end of *Gag* and the whole protease coding region. PCR products from the same sample were pooled, cloned into pGEM T-Easy vector System II (Promega) and transformed into *E. coli* competent cells. Twenty recombinant plasmids were used per time point to determine the proportion of mutant *vs*. WT virus in each competitive culture time points. Genotyping was performed with d-Rhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and subsequent electrophoresis in an automated sequencer (either an ABI PRISM 310 or 377; Applied Biosystems).

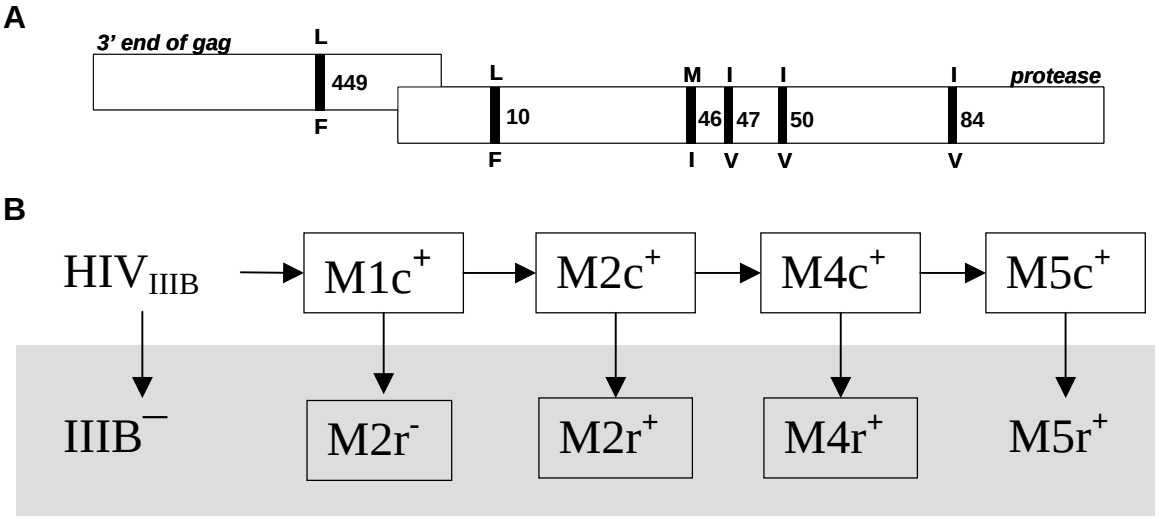
***Protein maturation analysis***. Virion associated PR activity was determined by Western blot analysis using Gag specific antibodies. After budding from the cell, virion maturation is completed through the cleavage of the Gag poliprotein by the viral protease into the structural proteins, matrix, capsid and nucleocapsid. For sample preparation, the viruses were collected by centrifugation and the pellet lysed directly in loading buffer and analyzed by SDS-PAGE. The Gag cleavage products were visualized on the Western blots using antibodies against capsid protein, p24. Defects in PR activity result in the accumulation of p41, a partially cleaved precursor, which was quantified and expressed as a percentage of Gag intermediates and compared with the reference virus ($HIV_{NL4-3}$).

***Statistical analysis***. All statistical analyses were performed in the R language (http://www.r-project.org).

## RESULTS

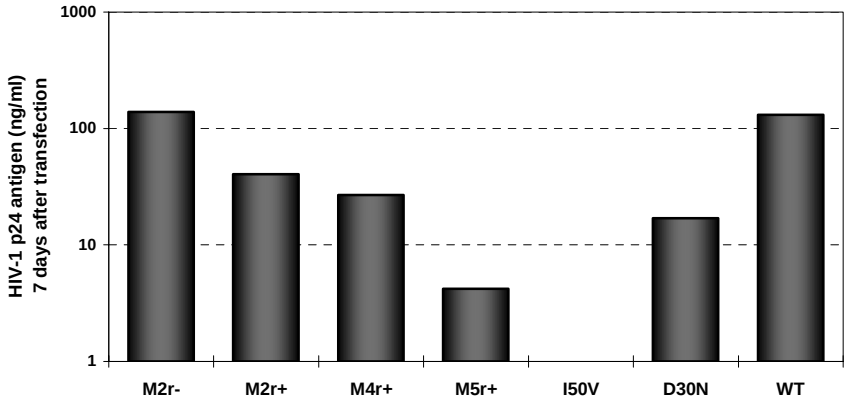
### *Production of recombinant viruses.*

Each recombinant virus vector produced viruses, except for the protease single mutant I50V which had been constructed by site directed mutagenesis and confirmed by DNA sequencing (Fig. 2A). The recombinant virus M5r$^+$ produced virus after transfection, but did not replicate to a titer sufficient to set up competitive cultures. We also detected a genotypic change in M2r$^-$ respect to its parental virus M1c$^+$: M1c$^+$ had only the protease substitution L10F in presence of the (p1/p6) *Gag* cleavage site mutation L449F, while M2r$^-$ had the mutations L10F/I84V in absence of any *Gag* cleavage site mutation (Table 1 and Fig 1). This difference seems likely to be due to selection of a different virus in the process of molecular cloning from a culture that contained a mixed population of different mutants as well as the wild-type.

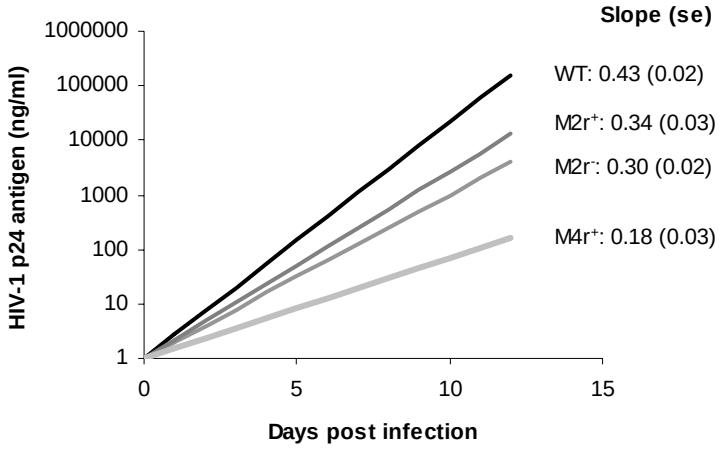



**(Fig 1)**. **A.Location of the drug-resistant mutations in the protease and the cleavage site mutations in Gag of the amprenavir selected viruses used in the study**. The single-letter code in the upper part of the figure represents the wild-type amino acid while the lower code indicates the substitution. Numbers indicate the amino acid position of the substitution in Gag and protease. **B.Flow chart showing the different mutants obtained during the study. Nomenclature according to Table 1**. Viruses in the shadowed area wereto Table 1. Viruses in the shadowed area were obtained by cloning the 3'-end of Gag and the protease in a Gag-protease defective vector with a HIV$_{NL4-3}$ backbone as described in methods. A recombinant virus assay was used to characterize virus within boxes, either selected virus pools (upper row) or cloned recombinant viruses (lower row).

**A**




**B**




**(Fig.2)**. **A.p24 antigen production after transfection**. 10 µg of plasmid DNA were electroporated in MT-2 cells and production of p24 was measured seven days after transfection. I50V mutant virus did not replicate in MT-2 cells after transfection. The replication-impaired D30N mutant, selected in presence of nelfinavir, is added here for comparative purposes. All mutants were constructed in a $HIV_{NL4-3}$ isogenic backbone. **B. Replication kinetics after infection**. MT-2 cells were infected with a MOI=0.001 and maintained in culture for 16 days. Recombinant virus production was monitored overtime by measuring p24 antigen production in supernatants fluids. Virus growth was plotted in a logarithmic scale showing continuous increase in the rate of virus production. To compare the slope of all mutants, y-intercept was normalized to one initial value. The rate of increase of p24 did not differ significantly between the mutants $M2r^-$ and $M2r^+$. The rate of p24 increase for $M2r^-$ and $M2r^+$ was approximately 75% that of wild-type, and the rate of p24 increase for $M4r^+$ was approximately 50% that of wild-type.

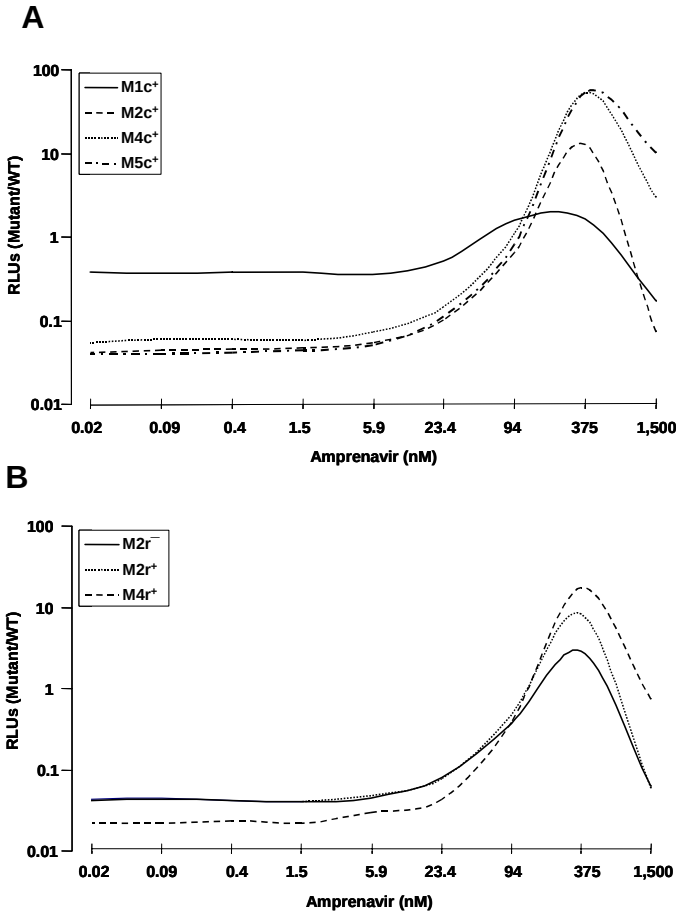
### *Phenotypic susceptibility of protease mutants.*

Drug susceptibility of the virus isolates selected in vitro during passage in increasing concentrations of amprenavir ("r" series), and the recombinant viruses derived from them ("c" series), were assayed against a panel of PIs (Table 1). Resistance to amprenavir increased as mutations accumulated. The acquisition of mutations M46I, I50V and subsequently I47V by an L10F/I84V virus increased the $IC_{50}$ from 8.4-fold ($M2c^+$) to 48-fold ($M5c^+$). Notably, amprenavir-resistant viruses also had a parallel loss of susceptibility to lopinavir. The $IC_{50}$ of this drug increased from 7.7-fold in $M2c^+$ to 31-fold in $M5c^+$. We also observed moderate cross-resistance to ritonavir (5 to 7-fold-increase in $IC_{50}$ for $M4c^+$, $M4r^+$ and $M5c^+$) and weak cross-resistance to nelfinavir, predominantly due to the low level of nelfinavir resistance ($IC_{50}$=4.1) in the $M5c^+$ strain. All drug-resistant mutants remained susceptible to saquinavir and indinavir. It should be noted that the only difference between $M2r^-$ and $M2r^+$, the cleavage site mutation L449F, increases PI resistance.

### *Viral replication capacity.*

Viral replication capacity of amprenavir-selected viruses in absence of drugs, was measured using several approaches, including a single-cycle replication capacity assay, replication kinetics as measured by the production of Gag p24 antigen in parallel cultures, as well as competitive virus replication. Based on the single-cycle replication capacity assay amprenavir-selected virus isolates and their cloned recombinant viruses exhibited <10% of the replication capacity of the wild-type reference strain (HIV-$1_{NL4-3}$) in the absence of drug (Table 1). There were significant differences between the replication capacities of the different amprenavir-selected strains. The replication capacity of $M1c^+$ was significantly higher(35.9%; linear mixed effects model, P<0.0001) and the replication capacity of $M4r^+$ was significantly lower (2%; P = 0.03) than the rest of the strains($M2c^+$, $M4c^+$, $M5c^+$, $M2r^-$, $M2r^+$: range 4-6.3%) in the absence of drug (Table 1). Replication capacity data was also obtained in the presence of different amprenavir concentrations by a different analysis of the drug susceptibility assays. Although the

viruses with higher amprenavir $IC_{50}$s had a relative replication advantage over wild type in higher amprenavir concentrations, the same rank order of relative replication capacity of these mutants was seen in lower amprenavir concentrations as in the absence of drug (Fig 3).

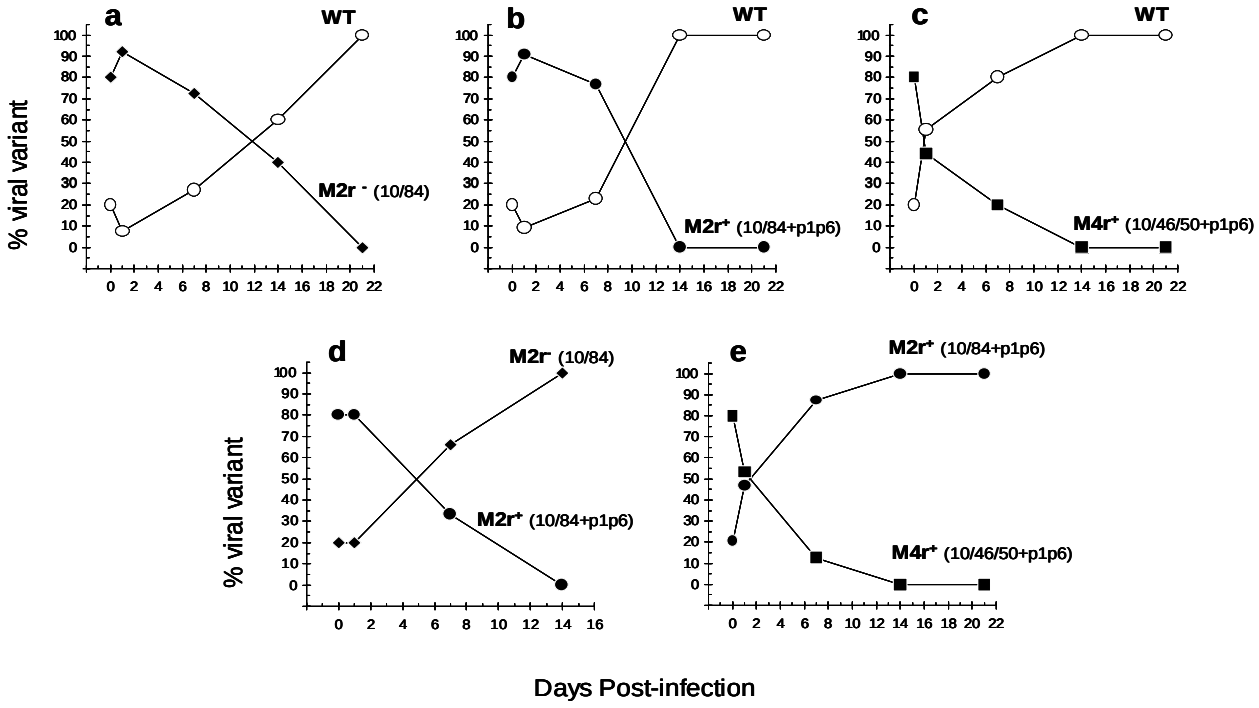



**(Fig 3). Replication capacity as a function of amprenavir concentration.** The ratio of mutant to wild-type relative luciferase units (RLU) was determined in the presence of amprenavir dilution series. **A.** Amprenavir-selected mutants directly obtained from culture supernatant fluids ($M1c^+$, $M2c^+$, $M4c^+$, $M5c^+$), and **B** recombinant mutants($M2r^-$, $M2r^+$, $M4r^+$).

Gag polypeptide processing of viruses produced in the single cycle assay was evaluated by determining the accumulation of a p41 (MA/CA) intermediate that precedes production of the fully processed p24 CA (Table 1). The p41 in virions increased from 7% in M1c$^+$ to 21% in M5c$^+$, relative to the reference virus. The accumulation of the incompletely processed Gag p41 peptide correlated with the increase in $IC_{50}$ to amprenavir and lopinavir (Spearman's rank correlation coefficient = 0.87, P=0.02), although there was no significant difference in replication capacity as measured using the single cycle assay. Replication capacity of these mutants in the absence of drug was also determined by the kinetics of Gag p24 antigen production (Figure 2B). The rate of increase of p24 did not differ significantly between HIV-$1_{NL4-3}$ and IIIB$^-$ (data not shown) and between the mutants M2r$^-$ and M2r$^+$. Overall, the rate of p24 increase for M2r$^-$ and M2r$^+$ was approximately 75% that of wild-type, and the rate of p24 increase for M4r$^+$ was approximately 50% that of wild-type. This assay suggested the order (in decreasing replication capacity): IIIB$^-$ > (M2r$^-$, M2r$^+$) > M4r$^+$, consistent with the results of the single-cycle assay.

A rank order of replication capacity among the recombinant viruses in the absence of drug was also determined using competition cultures. Each mutant virus was competed against a wild-type reference virus (IIIB$^-$) and each of the other mutant viruses. At least 20 molecular clones were sequenced to measure the relative frequency of WT *vs.* mutant virus at days 1, 7, 14 and 21 of each competitive culture. We found a progressive decrease in the relative replication capacity of recombinant virus clones, in the absence of drug, corresponding to viral isolates selected in vitro at increasing concentrations of amprenavir (Fig 4). The replication of M4r$^+$ was the most impaired based on competition with WT and M2r$^+$ viruses. M2r$^+$ replicated less well than the WT or M2r$^-$ viruses. These competition results in the absence of drug were consistent with the single cycle assay data and the p24 growth kinetics; however, in the competitive cultures the M2r$^-$ had a higher replication capacity than M2r$^+$.

Site directed mutagenesis was done to introduce the mutation I50V in pNL4-3. Recovered clones were sequenced and presence of the I50V as unique change in Gag and PR was confirmed. Three independent clones were transfected three different times in MT2 cells in presence of adequate positive controls. The cultures never produced detectable amounts of p24 antigen. In an independent laboratory (ViroLogic) similar results were obtained with a totally independent procedure using different site directed PR 50 mutant clones (N. Parkin and R. Ziermann, unpublished observation). The data obtained from all of experiments allowed us to define the following replication capacity rank: WT > M1c$^+$ > M2r$^-$ >M2r$^+$,c$^+$>M4r$^+$,c$^+$>M5c$^+$>I50V.




**(Fig 3). Replication capacity in competitive cultures. Clonal analysis of mixed competitive infections of MT-2 cells with drug-resistant HIV-1 mutants selected by amprenavir**. Coinfections were carried out with unequal amounts of PI-resistant mutants versus either WT ($HIV_{NL4-3}$) or another mutant. The proportion of each virus is plotted over time. **(a)** WT *vs.* M2r$^-$, **(b)** WT *vs.* M2r$^+$, **(c)** WT *vs.* M4r$^+$, **(d)** M2r$^-$ *vs.* M2r$^+$, and **(e)** M2r$^+$ *vs.* M4r$^+$. WT is the IIIB$^-$ virus, as described in metthods.

**TABLA.1**

| Virus | protease mutations | 3' end Gag cleavage site mutations | Drug susceptibility[a] (Fold change in $IC_{50}$) | | | | | | Replication capacity[b] (% relative to wt) | Gag processing[c] (% of p41) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | APV | LPV | RTV | NFV | IDV | SQV | | |
| | | | | | | | | | | |
| **M1c$^+$** | L10F | L449F | 1.8 | 1.6 | 1.1 | 1.3 | 1.5 | 1.3 | 36 | 7 |
| **M2c$^+$** | L10F, I84V | L449F | **8.4** | **7.7** | 4.0 | 3.4 | 3.2 | 3.7 | 4 | 14 |
| **M4c$^+$** | L10F, M46I, I50V | L449F | **21** | **19** | **5.2** | 2.9 | 1.6 | 1.3 | **6** | 17 |
| **M5c$^+$** | L10F, M46I, I47V, I50V | L449F | **48** | **31** | **6.5** | **4.1** | 2.4 | 2.0 | 4 | 21 |
| | | | | | | | | | | |
| **M2r$^-$** | L10F, I84V | — | 3.7 | 2.8 | 2.1 | 1.4 | 1.4 | 1.7 | 4 | 16 |
| **M2r$^+$** | L10F, I84V | L449F | **4.6** | **6.2** | 3.6 | 2.6 | 2.1 | 2.7 | 4 | 14 |
| **M4r$^+$** | L10F, M46I, I50V | L449F | **18** | **15** | **5.0** | 3.3 | 1.2 | 1.4 | 2 | 17 |
| **M5r$^+$** | L10F, M46I, I47V, I50V | L449F | ND[d] | ND | ND | ND | ND | ND | ND[d] | ND |

Drug susceptibility of amprenavir-resistant to a panel of HIV-1 protease inhibitors. Amprenavir-selected mutants directly obtained from culture supernatant fluids (M1c$^+$, M2c$^+$, M4c$^+$, M5c$^+$), and recombinant mutants (M2r$^-$, M2r$^+$, M4r$^+$), were assayed for susceptibility to a panel of multiple PIs: APV, amprenavir; LPV, lopinavir; RTV, ritonavir; NFV, nelfinavir; IDV, indinavir; and SQV, saquinavir. In this assay, drug susceptibility is considered to reduce when there is a 2.5-fold increase in $IC_{50}$ with respect to the reference strain. This threshold is applied for all protease inhibitors except lopinavir which cut-off has been redefined to 10-fold based on clinical trial data. The range of reproducibility of this assay for protease inhibitors is <2-fold [30]. [b] Viral replication capacity relative to the wild-type was determined by a modification of the phenotypic single-round replication assay. [c] Biochemical analysis of the accumulation of p41MA-CA (p17-p24) in the virions as a result of partial/incomplete protease processing of p55Gag. Values were normalized towild-typevirus. [d] ND, not determined due to low virus after transfection.

## DISCUSSION

We evaluated the protease inhibitor susceptibility and replication capacity of a series of HIV-1 variants that were progressively selected in vitro by serial passage of HIV-$1_{IIIB}$ in CEM-SS cells in presence of increasing concentrations of amprenavir [6]. Following seven passages, a variant with PRL10F/I84V mutation dominated the population. Two passages later the dominant variant contained L10F/M46I/I47V/I50V mutations. [6]. Viruses with a similar spectrum of mutations have been reported by others following selection in vitro by amprenavir [8], and has been observed in viruses from patients failing amprenavir treatment [7, 16].

In the present study, we used this series of amprenavir selected viruses, as well as recombinant virus clones derived from them, to characterize the protease inhibitor drug resistance/cross-resistance patterns and replication capacity of amprenavir-selected mutants.

The accumulation of two mutations in the protease (L10F/I84V), along with the gag L449F cleavage site mutation, resulted in moderate levels of resistance to amprenavir and lopinavir. Transitioning from the double protease mutant (L10F/I84V) to the triple (L10F/M46I/I50V) or the quadruple (L10F/M46I/I47V/I50V) protease mutants increased lopinavir cross-resistance from 7.7 to a 19 and 31-fold change in $IC_{50,}$ respectively. This observation is discordant with the current genotypic rule, which specifies multiple protease mutations (6 or more) are needed to confer at least 10-fold resistance to lopinavir [17]. Similar drug-susceptibility results were obtained for drug selected virus populations ($M1c^+$, $M2c^+$, $M4c^+$ and $M5c^+$) and molecular clones derived from each population ($M2r^-$, $M2r^+$ and $M4r^+$). Intermediate levels (<10-fold) of cross-resistance to ritonavir and nelfinavir were also observed after accumulation of three mutations in the protease. Less cross-resistance was observed to saquinavir and indinavir, in agreement with previous studies [6]. These data warrant consideration, based on resistance testing, of saquinavir/ritonavir and indinavir/ritonavir, as well as lopinavir/ritonavir, as components of salvage regimens for patients who have failed a previous regimen containing amprenavir with virus genotypes

such as those studied here. However, the current data identifying new HIV mutation patterns conferring cross-resistance to lopinavir point out the need for further studies correlating genotype and phenotype before relying only on genotyping for prediction of cross-resistance to lopinavir in clinical specimens.

The data presented here are consistent with a recent report that 59% of the subjects who had taken amprenavir-based regimens (ACTG 347 [18]) and switched to a 4-drug regimen (indinavir, nevirapine, stavudine and lamivudine) achieved subsequent durable virological suppression after one year of follow- up [19]. Whether or not the mutation patterns associated with low replication capacity in this study were frequent in protocol ACTG347 and resulted in a better response to a salvage treatment including indinavir requires further investigation.

The relative replicative capacity of the amprenavir-selected mutants in the absence of drug was examined using several different methods: a single-cycle replication assay, which measures luciferase production or the accumulation of partially process Gag (p41), replication kinetics, and replication competition. The single-cycle replication capacity assay revealed large replication impairment (>90%) for all mutants tested except for M1c$^+$ (L10F) which had a replication capacity of 36% respect to the wild-type virus. This assay proved to be a rapid and reproducible method for measuring replication capacity that can also be done in presence of drug as well. Although the viruses grew better in the presence of high concentrations of amprenavir as more mutations accumulated, the same relative ranking of replication capacity (diminishing replication capacity as mutations accumulated) was seen in either low concentrations of amprenavir or in the absence of drug. In this assay the accumulation of partially processed Gag p41 (i.e. impaired PR activity), increased with the number of protease mutations (M5c$^+$ > M4c$^+$ > M2c$^+$ > M1c$^+$ > WT). The very low replication capacity values in the single cycle assay contrast with the fairly good replication in other culture conditions (such as in Fig 2's p24 production assay). The clinical relevance of the single cycle assay is not yet established and it should not be assumed that

viruses with very low replication capacity in that assay will not replicate in vivo.

Subtle differences in replication capacity were more precisely defined by repeated testing in culture-based assays. A clear ranking of decreasing replication capacity was obtained by measuring p24 antigen production after transfection with normalized amounts of DNA. p24 antigen production kinetics after infection with normalized $TCID_{50}$s confirmed the spectrum of replication capacity from wild-type to $M4r^+$ and was in agreement with previous observations [20]. Competitive cultures, despite being more laborious and time consuming, established an accurate ranking of replication capacity in absence of drug (WT > $M2r^-$ > $M2r^+$ > $M4r^+$) by allowing direct comparison of each mutant against the wild-type or other mutants. PR mutations were neither gained nor lost during the course of the assay. Recombinant virus clones ("r" series) were used when measuring replication capacity in culture-based assays, to eliminate the outgrowth of minor variants present in the pool of drug-selected isolates ("c" series) that might complicate the competition in absence of drug (data not shown). Such outgrowth of minor wild-type virus has been observed in vivo during treatment interruptions in HIV-1 infected patients with treatment failure [13, 21]. The use of virus infectivity titrations, rather than virion particle counts, to measure inocula of the two variants in the competition cultures likely underestimated replication capacity differences since a larger number of the less replicative virions would have been used. Nevertheless, differences in replication capacity were observed despite this more stringent methodology.

The PI susceptibility of the I50V mutant could not be tested because this virus did not replicate (Fig. 2a). Other authors have shown that $HIV\text{-}1_{IIIB}$ harboring the single substitution I50V remains susceptible to amprenavir in culture [6]. The inability of the I50V virus to replicate in this system may relate to the different HIV-1 genetic background or the cells used [22]. We speculate that a PR I50V single mutant in the HIVNL4-3 background is unable to replicate.

In this study, the progressive accumulation of PR mutations did not re-establish viral replication capacity. This is in contrast with earlier studies of PI selected mutants, in which later mutations were seen to compensate for the replication capacity impairment associated with the first mutation [10, 23]. However, the shift from a L10F/I84V viral population to viral variants containing several mutations including I50V, suggests that PR L10F, M46I and the Gag L449F may work as compensatory mutations for PR I50V. Accumulation of PR I47V further increased amprenavir and lopinavir resistance but reduced viral replication capacity. Biochemical studies have also shown that the addition of M46I and I47V improves the replication of I50V mutant viruses [24]. PR M46I has been suggested to play a compensatory role in resistance development [25].

Notably, the only difference between $M2r^{-}$ and $M2r^{+}$ was the accumulation of Gag L449 in the P1' residue of the p1/p6 cleavage site. This cleavage site mutation decreased amprenavir and lopinavir susceptibility of the L10F/I84V virus from 3.7 and 2.8-fold to 4.6 and 6.2-fold, respectively. This Gag substitution had been previously reported to reduce susceptibility to the protease inhibitors lopinavir or BILA 1906 BS, but not to saquinavir or ritonavir [26-28]. This cleavage site mutation incompletely restored virus replication capacity of an amprenavir-selected isolate [29].

In summary, amprenavir-selected viruses with fewer than five mutations can exhibit >10-fold increase in $IC_{50}$, to amprenavir, and also to lopinavir. This suggests that a given number of resistance mutations in protease may not be an adequate criterion for lopinavir resistance and suggests that lopinavir/ritonavir use, after an amprenavir-containing regimen fails, may be better guided by phenotypic resistance testing. This approach, however, will be limited by the possibility that some clinical specimens may not be able to be phenotyped. The single-cycle growth assay for replication capacity yielded similar results as competitive cultures in absence of drug, although small relative differences were more apparent with competitive cultures. These data suggest further study of cross-resistance to lopinavir in speci-

mens from patients failing amprenavir and provide initial data on concordance between a rapid single-round growth assay and a slower, more laborious measure of replication capacity.

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# CAPÍTULO III

## Relative replication fitness of multi-nucleoside analogue resistant HIV-1 strains bearing a dipeptide insertion the fingers subdomain of the reverse transcriptase and mutations at codons 67 and 215

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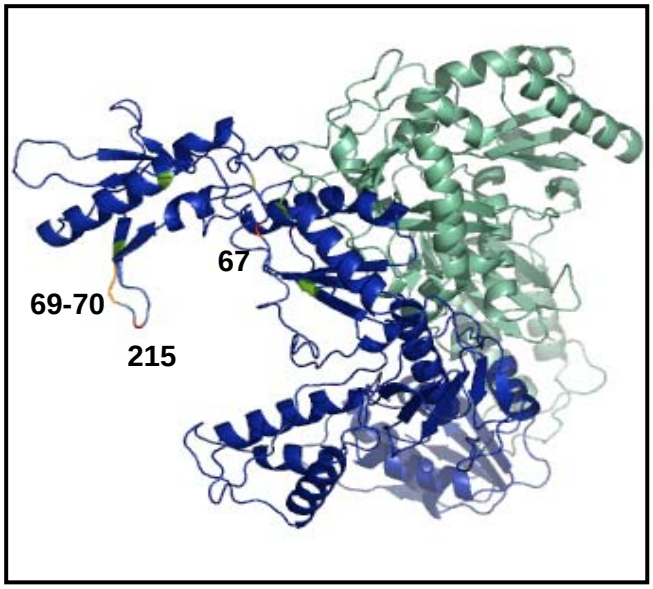
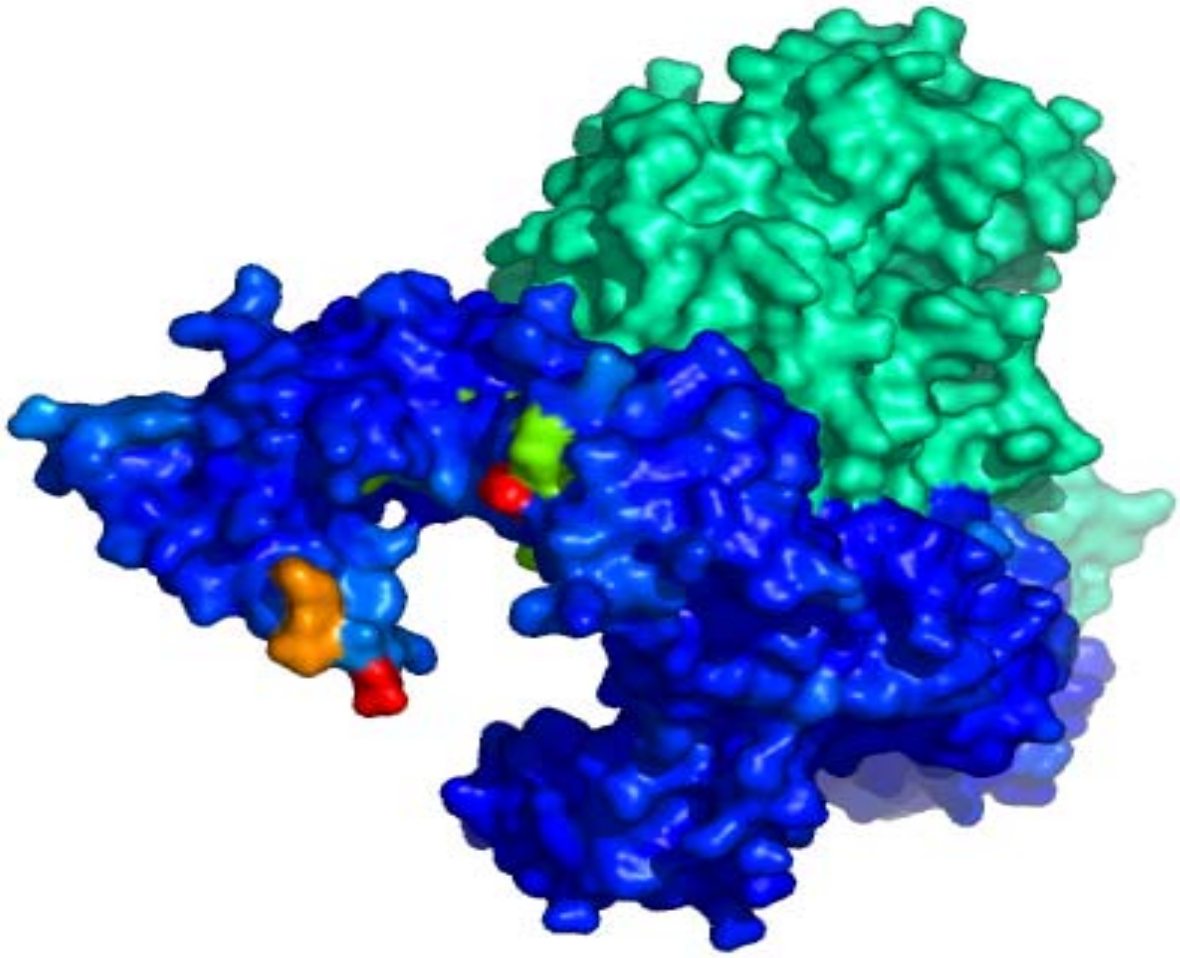


## Transcriptasa Inversa del VIH-1

Estructura de la TI cristalizada, resolución 2,60 angstroms; J.Ren y cols. 1995. Modificada en Pymol. En azul subunidad p66 con actividad polimerasa dentro de esta, en naranja aparecen representada la zona de la inserción entre los aminoácidos 69-70, las posiciones retromutadas representadas en rojo (215, 67); en verde aparecen las posiciones de las mutaciones de resistencia presentes en la TI del paciente. En azul cian subunidad p51 con actividad RNasa H.

La inserción de dos serinas en la posición 69 de la TI del VIH-1 es una de las rutas mutagénicas por las cuales el VIH-1 alcanza multirresistencia a los análogos a nucleósidos, en el contexto de una secuencia con múltiples mutaciones de resistencia a la zidovudina (pe. M41L, L210W, T215Y). Partiendo de la muestra de plasma de un paciente con este perfil de multirresistencia, hemos evaluado la capacidad replicativa de diversos virus recombinantes respecto de una cepa de referencia. Los virus se caracterizan por haberles introducido las mutaciones D67N, Y215T, Y215S o Y215N en el conjunto de las mutaciones presentes en el genoma del virus del paciente. Los resultados obtenidos nos indican que la mutación D67N tiene poco impacto en capacidad replicativa y en el perfil de resistencias a análogos; por el contrario los cambios en la posición 215 de Y→T, Y→S o Y→N tienen un efecto mucho más marcado. La reversión en la posición 215 de Y→T, Y→S o Y→N es suficiente para hacer recuperar al virus su capacidad replicativa en cultivo en ausencia de fármaco y, de un modo paralelo, la susceptibilidad a los análogos zidovudina y estavudina. La posición 215 de la TI, por lo tanto, juega un papel clave tanto en capacidad replicativa como a la hora de conferir resistencias a análogos en virus multirresistentes con la inserción 69SS.

## ABSTRACT

A two-serine insertion at position 69 (i69SS) of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) appears to be critical to enhance multinucleoside RT inhibitor resistance (MNR) in the sequence context of multiple zidovudine (AZT) resistance mutations (i.e. M41L, L210W, T215Y). In this study we measured the replication capacity relative to the wild-type (WT) HIV-1 of a series of recombinant viruses carrying the i69SS in the background of a clinical isolate with MNR, in which we introduced mutations D67N, Y215T, Y215S or Y215N. In vitro measurements included replication kinetics and growth competition assays at different multiplicities of infection. While the addition of D67N had a minor effect on replication capacity, the reversion of Tyr-215 to Thr, Ser or Asn was sufficient to increase the virus ability to replicate in a drug-free environment. The same genotypic changes at position 215 rendered the MNR virus susceptible to AZT and stavudine. Interestingly, the presence of the insertion together with mutation T215Y in an otherwise WT sequence background was not sufficient to confer high-level resistance to AZT, although its replication capacity was clearly impaired. Therefore, the RT residue 215 plays a critical role in both replication capacity and drug resistance of multidrug-resistant viruses containing the i69SS.

## INTRODUCTION

Human immunodeficiency virus type (HIV-1) infection is currently treated with highly active three-drug combination therapies. However, emergence of drug resistance-associated mutations is the major limitation to long-term treatment efficacy. Moreover, the fact that the 20 currently approved HIV-1 inhibitors for clinical use belong to only four different drug-classes facilitates the appearance of cross-resistance among different drugs of the same family. The nucleoside analogue reverse transcriptase inhibitors (NRTI) were the first type of compounds used for HIV treatment and are still part of almost all therapeutical regimens. Except for lamivudine, high-level resistance to NRTI is associated with multiple mutations in the reverse transcriptase (RT). Certain mutational pathways lead to the selection of RTs with Multi-NRTI resistance (MNR). One of those patterns involves an insertion of two amino acids (often Ser-Ser, Ser-Gly or Ser-Ala) between residues 69 and 70 of HIV-1 RT(Briones et al., 2000; Briones and Soriano, 1999; De Antoni et al., 1997; de Jong et al., 1999; Larder et al., 1999; Ross et al., 1999; Sugiura et al., 1999; Tamalet et al., 1998; Winters et al., 1998). The insertion at position 69 has been described in about 2% of HIV-1-infected patients that received prolonged therapy with zidovudine (AZT), together with (or followed by) administration of other NRTIs (Van Vaerenbergh et al., 2000). The insertion is located in the ß3-ß4 hairpin loop at the fingers subdomain of the RT, and appears to be associated with multiple amino acid substitutions, including AZT-resistance mutations, such as T215Y. By themselves, the insertions cause low-level resistance to each of the NRTIs (Larder et al., 1999; Lennerstrand, Stammers, and Larder, 2001; Mas et al., 2000; Winters et al., 1998),and drug resistance tests of recombinant patient isolates containing insertions together with T215Y/F and other AZT-resistance mutations showed reduced susceptibility to all NRTI (Dunne et al., 2001; Hertogs et al., 2000; Larder et al., 1999; Mas et al., 2000; Masquelier et al., 2001; Petropoulos et al., 2000; Winters et al., 1998). Insertions between codons 69 and 70 have been also associated with resistance to tenofovir (Harrigan et al., 2002; White et al., 2004). The in-

sertion of two serines at codon 69 (i69SS) appears to be critical to enhance AZT and stavudine (D4T) resistance in the sequence context of a multidrug-resistant HIV-1 RT (Dunne et al., 2001; Hertogs et al., 2000; Larder et al., 1999; Mas et al., 2000; Masquelier et al., 2001; Petropoulos et al., 2000; Winters et al., 1998). Phenotypic resistance to those thymidine analogues correlates with an increased ATP-dependent phosphorolytic activity that facilitates the efficient removal of the 3'-terminal nucleotide from NRTI-terminated primers (Lennerstrand, Stammers, and Larder, 2001; Mas et al., 2000; Mas et al., 2002). In the absence of drugs, the insertion by itself does not confer any fitness disadvantage, when occurring in a WT sequence context (i.e. HIV-$1_{BH10}$). However, it contributes to increase viral fitness of an HIV-1 isolate harboring the MNR genotype to levels which are roughly similar to those observed with the WT HIV-$1_{BH10}$ strain (with or without the insertion) (Quiñones-Mateu et al., 2002). So far, two-amino acid- insertions between positions 69 and 70 has only been reported once in patients with primary HIV-1 infection (Daar et al., 2002). Their low prevalence in HIV-treated patients suggests that its transmission fitness is also low. In this work, we have measured the effect of mutations at codons 67 (D67N) and 215 (Y215T, Y215S and Y215N) on the phenotypic susceptibility to NRTI and replication capacity of recombinant HIV-1 variants carrying the i69SS in the sequence background of a clinical multidrug-resistant isolate (Table 1).

## MATERIALS AND METHODS

***Patient sample***. The plasma sample was obtained from a heavily treated HIV-1 infected individual as part of a previous study that screened HIV-1-infected patients for the presence of virus with insertions in the fingers subdomain of HIV-1 RT (Briones and Soriano, 1999). The donor was an HIV-infected 38-year old man, who had been extensively treated with several RT and protease inhibitors, including an initial treatment with AZT in monotherapy (1992 to 1996) and a current regimen at the time of plasma withdrawal combining five antirretroviral drugs (D4T, dideoxyinosine, nevirapine, saquinavir and nelfinavir). Viral RNA was extracted from plasma and the RT-coding region was RT-PCR-amplified and cloned as previously described (Briones et al., 2000; Mas et al., 2000). Sequencing of the HIV-1 *pol* gene showed that apart from the insertion of two serines between codons 69 and 70, it contained 43 additional mutations scattered throughout the entire RT-coding sequence, including 10 amino acid substitutions related to drug resistance (D'Aquila et al., 2003).

***Generation of Recombinant Viruses.*** The recombinant viruses containing the RT-coding regions of a WT HIV-1 strain (BH10) or of the MNR HIV primary isolate mentioned above (SS) were constructed as previously described (Mas et al., 2000). Mutations D67N (SS/67N), Y215T (SS/215T), Y215S (SS/215S), Y215N (SS/215N) were introduced by site-directed mutagenesis in a plasmid carrying the nucleotide sequence encoding the 66-kDa subunit of SS RT (Matamoros et al., 2004) (Table 1). In addition, mutation T215Y was introduced in a plasmid encoding a mutated p66 subunit of BH10 RT, which contained the T69SSS insertion (Mas et al., 2000), to obtain the mutant SSSY (Matamoros et al., 2004) (Table 1). A PCR-amplified fragment containing the full-length RT-coding region was cotransfected with an RT-deleted HXB2-D clone in SupT1 cells [95]. Harvested viruses were propagated in MT-4 cells. The 50% tissue culture-infectious dose ($TCID_{50}$) in each virus stock was determined in the same cell line by using the method of Reed and Muench (Reed and Muench, 1938).

**(Table.1)Amino acid sequence differences between RTs from WT HIV-1 (BH10 strain) and recombinant virus derived from the multidrug-resistant clinical HIV-1 isolate (SS).**

| | 41 | 62 | 67 | 69 | | | 70 | 108 | 118 | 181 | 184 | 210 | 215 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT (BH10) | M | A | D | T | | | K | V | V | Y | M | L | T |
| SSSY | - | - | - | S | S | S | - | - | - | - | - | - | Y |
| SS | L | V | - | S | S | S | R | I | I | C | I | W | **Y** |
| SS/215S | L | V | - | S | S | S | R | I | I | C | I | W | **S** |
| SS/215N | L | V | - | S | S | S | R | I | I | C | I | W | **N** |
| SS/215T | L | V | - | S | S | S | R | I | I | C | I | W | **-** |
| SS/67N | L | V | **N** | S | S | S | R | I | I | C | I | W | **Y** |

Only those amino acid differences found at codons associated with resistance to RTs are indicated [94]. Six different plasmid constructions were generated containing RT sequences from the wild-type strain (BH10) and the sequence found in the clinical isolate (SS). The mutation G333E was also present in SS and mutants derived from this construct.

***Phenotypic Analysis***. Phenotypic susceptibility to nucleoside RT inhibitors of the new HIV variants was tested *in vitro* by the MTT assay (Pauwels et al., 1988). Phenotypic resistance was determined by measuring fold-increase in 50% inhibitory concentration ($IC_{50}$) values compared with the reference WT HIV-$1_{HXB2}$ strain, carrying the RT-coding region of BH10.

***Analysis of Replication Kinetics***. Inocula of 5,000 $TCID_{50}$ of each virus were used to infect 5 X $10^6$ MT-4 cells (multiplicity of infection of 0.001) as previously described (Martinez-Picado et al., 1999; Prado et al., 2002). After incubation for 2 h at 37°C, cells were washed twice with phosphate-buffered saline and resuspended in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (Sigma), 10 mM HEPES buffer (Invitrogen), 50 mg of penicillin/ml and 50 mg of streptomycin/ml (Invitrogen) at a concentration of 0.5 X $10^6$ cells/ml. Duplicates of five-milliliter cultures were incubated in 6-well tissue culture plates (Nunc) in the absence of drugs. Supernatants (200 µl) from each culture were collected every day, and then an equal volume of culture media was added. Levels of p24 antigen were quantitated in cell-free

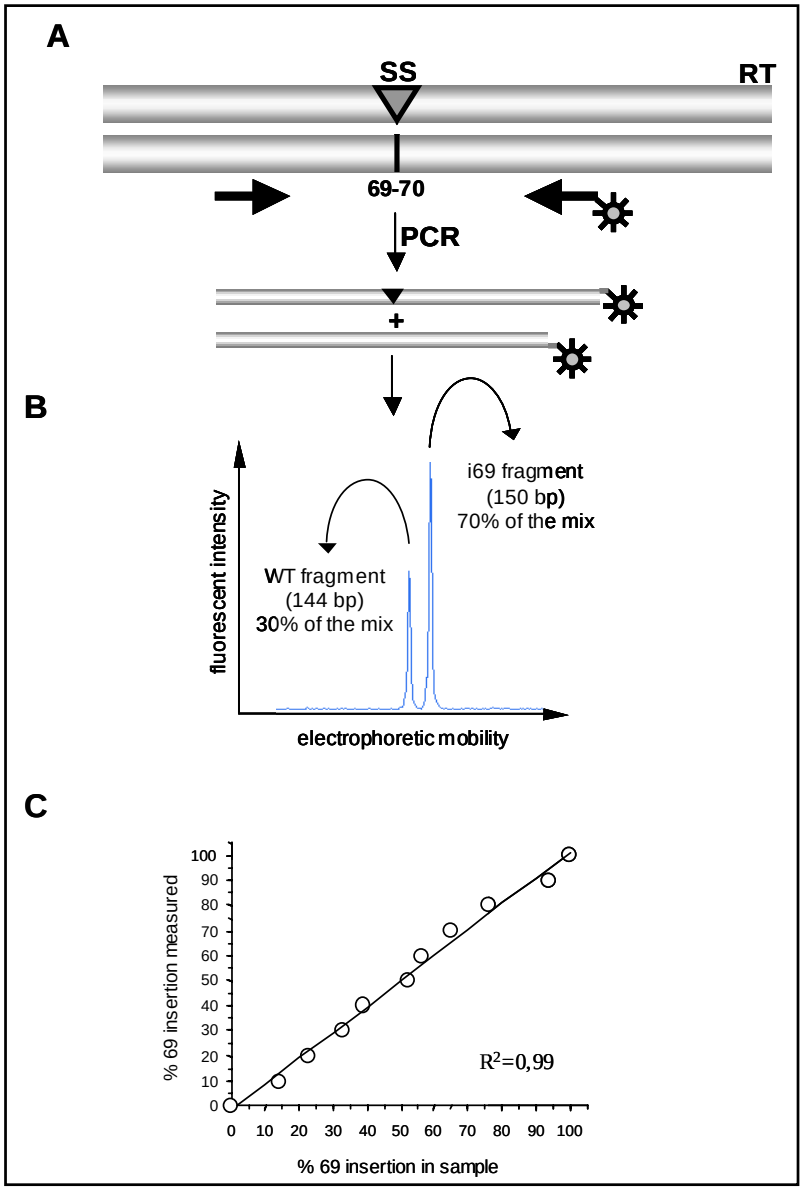
culture supernatants by using the HIV-1 p24 antigen assay (Innogenetics) and were used to monitor replication kinetics. Growth kinetics based on p24 production were analyzed by fitting a linear model to the log-transformed p24 data from the replicates for each mutant by maximum likelihood methods (Martinez-Picado et al., 2000; Prado et al., 2002). For a given mutant, the growth rate based on p24 production was assumed to be the same between replicates, but the initial level of p24 was allowed to vary between replicates according to a normal distribution. Missing values were assumed to be missing at random. This model was found to give a good fit to the data.

***Analysis of Relative Replicative Fitness in Virus Mixtures***. Growth competition assays were performed in MT-4 cells in absence of drug as described elsewhere (Martinez-Picado et al., 2000; Prado et al., 2002). Multiplicities of infection (MOI) of 0.01 and 0.001 were assayed. Infections were initiated with unequal amounts of two competing virus variants, according to virus infectivity titrations. Unequal ratios were used based on the rationale that an increase in proportion of the initially less abundant virus suggests a relatively better replicative capacity than the competing, initially more abundant virus, although random factors cannot be excluded with either unequal or equal starting ratios. Five-milliliter cultures were maintained in 6-well tissue culture plates at 0.5 X $10^6$ cells/ml. At day 3 of the first passage, half of the culture was replaced with fresh medium. At day 7, fresh MT-4 cells were reinfected with a dilution 1:100 of the supernant and cultured at 0.5 X $10^6$ cells/ml. Three additional passages were performed in the same way. Aliquots were removed from the cultures at days 3, 7, 10, 14, 17, 21, 24 and 28, centrifuged; total genomic DNA was extracted from the cells (Qiamp DNA blood Mini kit; Qiagen) for genotypic analysis (Fig. 1), and supernatants were stored at -80°C. Levels of p24 antigen were quantitated in cell-free culture supernatants of all time points to monitor replication kinetics of the competitions. The number of viable cells was also determined with trypan blue. Genotyping was performed at the end of each competition by using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and subsequent electrophoresis in an automated sequencer (ei-

ther an ABI PRISM 310 or 377; Applied Biosystems). MT-4 cells were also separately infected with mutants in the absence of WT virus to assess possible spontaneous reversion of the mutated codons.

***Length polymorphisms detection***. DNA was PCR-amplified in triplicate with primers RT2696-2725 (5'FAM - AAT TGG GCC TGA AAA TCC ATA CAA TAC TCC -3', where FAM is 6-carboxyfluorescein) and RT2839-2812 (5' - GGA TGT GGT ATT CCT AAT TGA ACT TCC C - 3') by using Platinum Taq High Fidelity polymerase (Invitrogen) and 25 thermal cycles (30 s at 94ºC, 30 s at 55ºC and 30 s at 68ºC). One µl of PCR products were mixed with 20 µl of formamide and one µl of the molecular size marker labeled with carboxytetramethyl rhodamine (Applied Biosystems) (Fig. 1A). DNA fragments were separated under denaturing conditions in a capillary electrophoresis analysis, and results were analyzed with the GeneScan software (Applied Biosystems), as previously described (Martinez-Picado et al., 2002). The area of each peak divided by the total area of the two peaks was used to determine the relative proportion of the two competing variants in each time point (Fig. 1B). Viral RNA was also extracted from the culture supernatants, reverse transcribed and PCR amplified in one step by using the OneStep reverse transcription-PCR kit (Qiagen) with the same primers indicated above.The reproducibility and robustness of the length polymorphisms detection method was tested by mixing known amounts of plasmids that contained the HIV-1 RT-coding region with and without the i69SS.

***Statistical analysis***. Statistical analyses were performed using the R language (http://www.r-project.org) and the SPSS programs version10.0.6 (SPSS Inc., Chicago, IL).




**(Fig.1) Schematic representation of GeneScan detection for growth competition experiments. (A)** HIV-1 DNA is extracted from the MT4 cells used in growth competition experiments. Samples are taken at different times after dual infection with two HIV-1 isolates (i.e. a WT recombinant clone and an i69SS-containing virus). Two primers, one of them labeled with FAM, were used to amplify an RT fragment by PCR.**(B)** Labeled PCR products were run in a capillary electrophoresis along with DNA size markers. Two peaks, each of them proportional to the relative among of the two viral variant in the original sample, appear at the 144 and 150 bp positions. **(C)** The sensitivity of the technique was assayed by mixing different proportions of two plasmids, one WT and another containing the 69 insertion in the RT. The detection is linear down to 10%. See Materials and Methods for experimental details.

## RESULTS

### *Resistance to nucleoside analogue inhibitors.*

Recombinant HIV-1 clones were assayed to establish the level of resistance to NRTIs in clinical use (Table 2). Within the context of a MNR genotype (including the i69SS) the addition of D67N, an AZT-resistance mutation, showed a minor effect in NRTI susceptibility. However, the reversion of Tyr-215 to Thr, Ser or Asn rendered the MNR virus susceptible to AZT and D4T (Table 2). These mutated viruses were also more sensitive to didanosine and zalcitabine inhibition. All recombinant viruses derived from the multidrug-resistant strain retained high levels of lamivudine resistance as a result of the M184I mutation. Interestingly, the presence of the insertion together with mutation T215Y in an otherwise WT sequence background conferred a moderate increase of AZT resistance.

### *Replication kinetics.*

The growth curves of all tested recombinant viruses were similar to the one shown by WT HIV-1 (Table 2). Moreover, differences observed between the WT and the virus showing the lowest replication capacity (i.e. SSSY) were not statistically significant.

**(Table.2) Susceptibility to nucleoside analogue reverse transcriptase inhibitors and replication capacity of HIV-1 constructs.**

| Virus | $IC_{50}$ (μM)[a] (fold-increase) | | | | | Replication Capacity[b] | |
|---|---|---|---|---|---|---|---|
| | AZT | d4T | ddI | ddC | 3TC | slope, *m*, (pg/ml·day)[c] | 95% C.I.[d] |
| WT (BH10) | 0.005 | 0.19 | 2.03 | 0.42 | 0.63 | 0.85 | 0.51-1.2 |
| T69SSS[e] | 0.002 | 0.26 | 1.91 | 0.14 | 1.84 | | |
| SSSY | 0.02 (4.0X) | 1.33 (5.5X) | 2.09 (1.0X) | 1.56 (3.7X) | 3.36 (5.3X) | 0.71 | 0.56-0.87 |
| SS | >7.48 (>1600X) | 2.60 (13.9X) | 17.64 (8.7X) | 5.37 (12.8X) | >20 (>34X) | 0.82 | 0.55-1.1 |
| SS/67N | 3.54 (708X) | 2.62 (14.0X) | 9.54 (4.7X) | 2.65 (6.3X) | >20 (>34X) | 0.86 | 0.64-1.1 |
| SS/215T | 0.007 (1.4X) | 0.49 (2.6X) | 11.61 (5.7X) | 2.41 (5.7X) | >20 (>34X) | 0.87 | 0.67-1.1 |
| SS/215S | 0.019 (4.1X) | 0.32 (1.7X) | 6.31 (3.1X) | 1.49 (3.5X) | >20 (>34X) | 0.87 | 0.71-1.0 |
| SS/215N | 0.006 (1.2X) | 0.29 (1.6X) | 4.93 (2.4X) | 1.29 (3.1X) | >20 (>34X) | 0.77 | 0.62-0.93 |

[a] The concentration of drug necessary to inhibit viral replication by 50% ($IC_{50}$) values represent the mean of 3 to 6 replicates. The fold increase in $IC_{50}$ relative to the wild-type virus control carrying the RT sequence of BH10 is shownbetween parenthesis.[b] In absence of drug.[c] Exponential growth constant or slope (*m*) derived from the equation log (y) = mt + log (b), where *y* is a measure of virus quantity (i.e. pg of p24 per ml), *t* is time in days and, *b* is the y-intercept.[d] 95% confidence intervals (C.I.).[e] $IC_{50}$ values taken from reference [84] for comparative purposes. This virus contained an $RT_{BH10}$ in which the codon 69 (Thr) has been replaced by three serines (T69SSS).

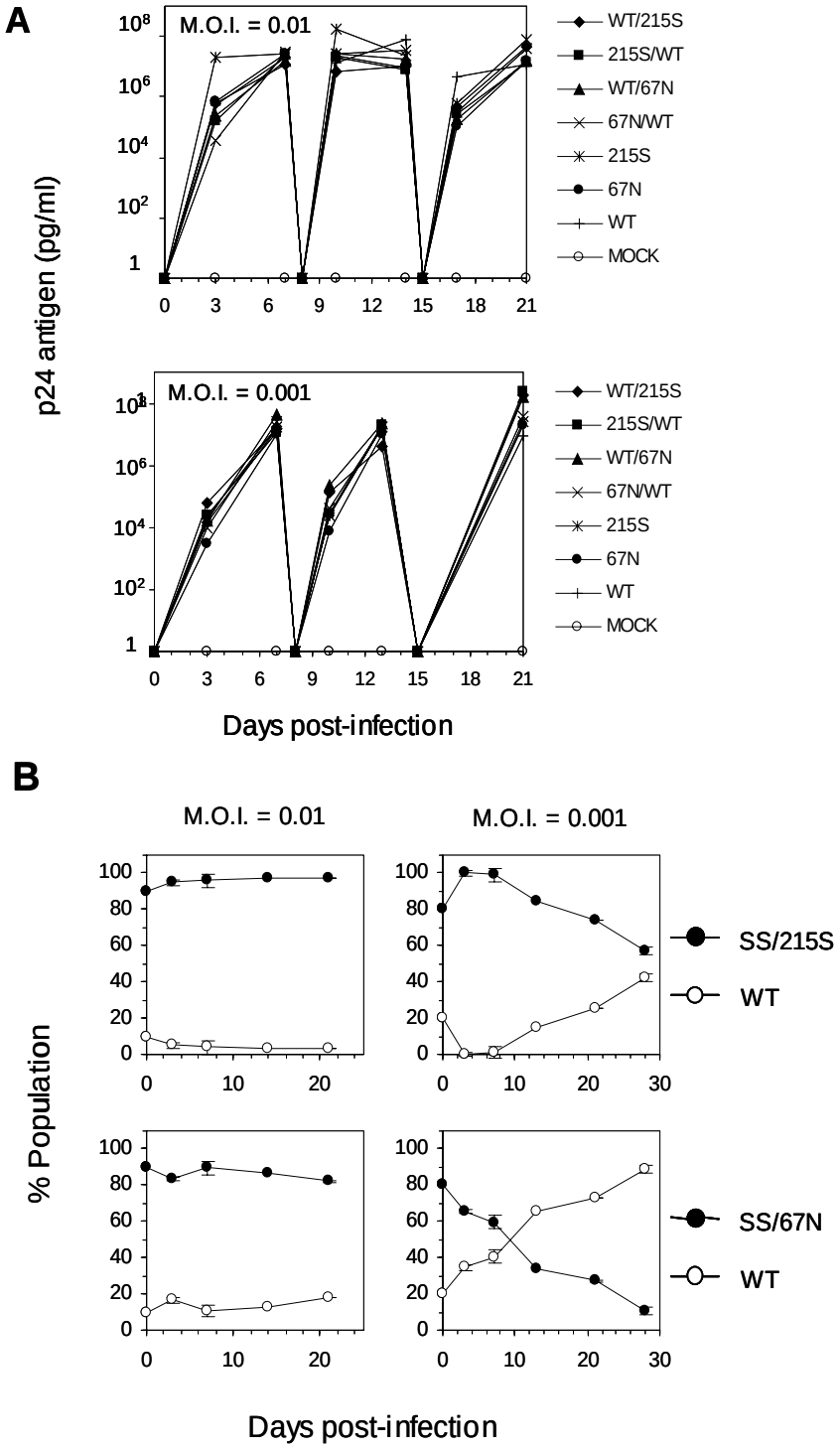
### *Relative Replicative Fitness in Virus Mixtures.*

In order to measure viral fitness differences more accurately, dual infections were performed in cell culture. Replication capacity was determined in vitro relative to the WT at different MOI over 28 days. The proportions of WT and i69SS-containing viruses were estimated by length polymorphisms detection methods (Fig. 1). Before applying this detection system to growth competition experiments, we set up the method with different mixtures of plasmids containing an HIV-1 RT fragment with and without the i69SS. Fig. 1C demonstrates an excellent agreement and reproducibility between the expected and the measured values of dual mixtures of sequences with and without the i69SS. The method was sensitive at least down to 10% of one of the viral strains.

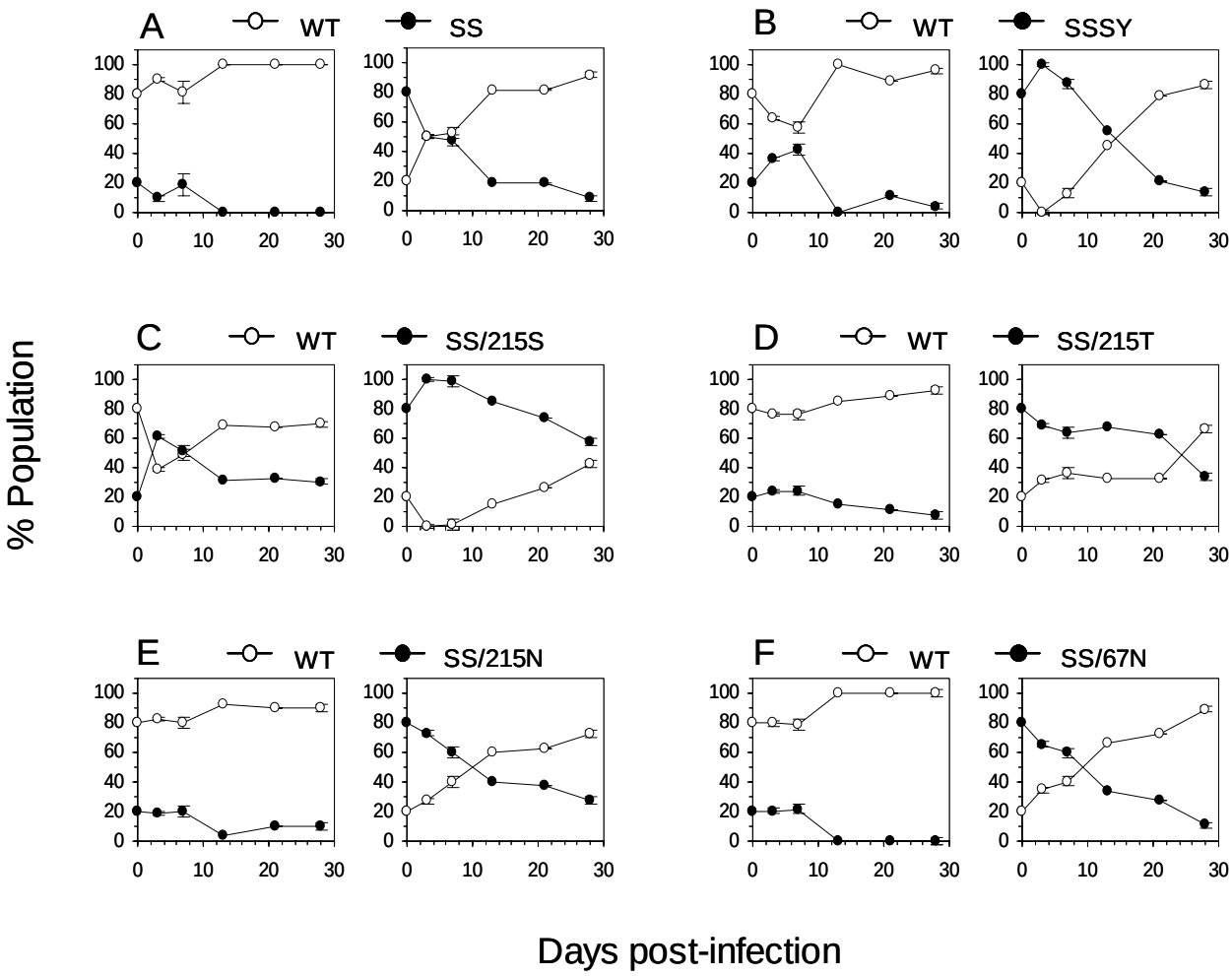
Competition growth assays were performed with different MOIs. We could observe differences in the outcome of the competitions between a MOI of 0.01 or 0.001 being the latter the one showing the most significant differences among the different viral strains compared. For example, at an MOI

of 0.01, fitness differences between the WT virus and mutant SS/215S were not significant. However, when the MOI was reduced to 0.001, the WT tended to outgrow the SS/215S (Fig. 2B). A similar behavior was observed in fitness competition assays with WT HIV-1 and mutant SS/67N. While at an MOI of 0.01, the mutant was slightly overgrown by the WT virus, at a lower MOI (0.001), the WT viruses had taken over the population by day 14 of culture (Fig. 2B). Interestingly, antigen p24 levels increased steadily during the first 6 days postinfection, at least during the first two passages, when infections were carried out at an MOI of 0.001 (Fig. 2A).

However, at higher MOIs (i.e. 0.01), maximum p24 levels were already detected within the first 3 days after infection in all assays. These results which were also consistent with simultaneous determinations of cell viability (data not shown) justify why the lower MOI is more efficient to distinguish subtle differences in fitness between the competing viruses.

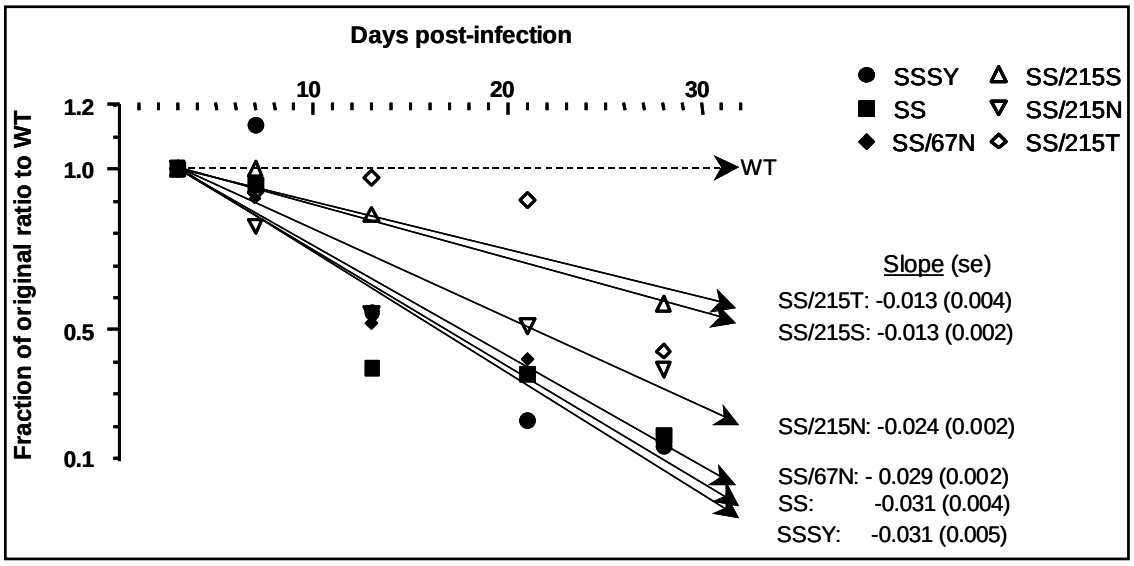



**(Fig. 2)**. **Influence of different MOIs in growth competition experiments**. **(A)** Antigen p24 production after HIV-1 infection of MT4 cells at an MOI of 0.01 (top panel) and 0.001 (bottom panel). Reinfection of fresh cells was performed every 7 days. **(B)** Changes in the evolution of WT HIV-1 *versus* mutants SS/215S or SS/67N in growth competition experiments set up at different MOIs.




**(Fig. 3). Replication capacity of different RT mutants containing the 69 insertion versus a WT**. HIV-1 strain assayed in growth competition experiments in MT4 cells and detected by GeneScan. Coinfections were carried out with nonequivalent amounts of mutants versus a WT HIV-1 strain. Two experiments, each initiated with a different proportion of the two viruses, are plotted in each panel. **(A)** SS *versus* WT; **(B)** SSSY *versus* WT; **(C)** SS/215S *versus* WT; **(D)** SS/215T *versus* WT; **(E)** SS/215N *versus* WT; **(F)** SS/67N *versus* WT. Open symbols refer always to the WT HIV-1 strain, and solid symbols refer to the HIV-1 RT mutants containing the 69 insertion. Proportions of different viral populations containing the 69 insertion in the RT were determined every 3 or 4 days. Error bars represent standard error of 3 independent measurements. Data on day 0 refer to input $TCID_{50}$ proportions.

All tested viruses had a replication capacity lower than the WT when assayed in a drug-free environment for an MOI of 0.001 (Fig. 3). Thus, even when infections were initiated with an excess of mutant virus (right panel of each competition in Fig. 3), the WT virus outgrew the mutant viruses SS, SSSY, SS/67N and SS/215N before day 15 of culture, while the shift occurred later for viruses SS/215S and SS/215T. In fact, the loss of fitness of these two viruses in comparison with the WT strain did not reach statistical significance. Fitness competition experiments gave consistent results, either when infections were initiated with an excess of mutant or with an excess of WT virus. Interestingly, the presence of the insertion together with mutation T215Y in an otherwise WT background (as in mutant SSSY) impaired fitness, rendering viruses with a replication capacity similar to that shown by the SS strain or the SS/67N mutant (Fig. 4). When the SS virus was used for comparison, SS/215T and SS/215S were significantly fitter ($p<0.05$) (Fig. 4). Recombination between the two input viruses was not detected by sequencing population-based PCR products at the end of the coculture.




**(Fig. 4) Determination of the relative replication efficiencies of recombinant HIV-1 strains containing the 69 insertion in the RT-coding region *versus* a WT virus, as determined from growth competition experiments**.

## DISCUSSION

A dipeptide insertion at position 69 in combination with T215Y/F and other thymidine-analogue-resistance mutations has been considered as a mutational pattern characteristic of MNR. This mutational pattern, however, is responsible for only a minority of resistant isolates: it is present in about 1% of the patients treated with at least one NRTI and 3% of the patients treated with more than three NRTIs (Stanford HIV RT and Protease Sequence Database; http://hivdb.stanford.edu; (Rhee et al., 2003). Previous reports described the role of the two serine insertion at positions 69/70 of the HIV-1 RT on NRTI resistance and the biochemical mechanism involved, as well as their influence on viral fitness in either a WT RT sequence background or in the context of a multidrug-resistant RT derived from a clinical isolate (designated as SS) (Mas et al., 2000; Mas et al., 2002; Quiñones-Mateu et al., 2002). In the present study we analyzed the effects on viral fitness and NRTI susceptibility of adding a new mutation in the β3-β4 hairpin loop at the fingers subdomain of the RT or reverting Tyr-215 to its WT genotype (Thr-215) or to other less common amino acids (i.e. Ser or Asn). Our results showed that the introduction of mutation D67N in the multidrug-resistant SS variant had no measurable effect on viral fitness or resistance to NRTIs. However, the reversion of Tyr-215 to Thr, Ser or Asn in the sequence context of a MNR background including the 69 insertion, rendered viruses susceptible to AZT and D4T, whose viral fitness increased in the absence of drug. Our results indicate that the RT residue 215 plays a critical role in both drug resistance and replication capacity of MNR viruses containing the i69SS. In the context of an MNR virus, the reversion of Tyr-215 to Thr that involves two nucleotide changes, or its substitution by Ser or Asn, which requires only one nucleotide substitution are sufficient to recover full susceptibility to AZT and D4T due to the loss of the ATP-dependent unblocking activity (Matamoros et al., 2004), as well as to increase the viral replication capacity in the absence of drugs. However, the interpretation of the data might be somehow limited by the use of a single primary isolate derived RT sequence.

It has been shown that, after discontinuation of AZT in patients harboring AZT-resistant strains of HIV-1 (i.e. isolates carrying mutations M41L, D67N, T215Y/F, K219Q/E, …), or in newly-infected individuals who had acquired an AZT-resistant virus by transmission, resistant variants were eventually replaced by AZT-susceptible revertants that often contained unusual amino acids at codon 215, such as Asp, Asn, Cys or Ser, through mutations involving one nucleotide change (de Ronde et al., 2001; García-Lerma et al., 2001; Yerly et al., 1998). These partial revertants appear to be fitter than the virus having Tyr-215 when tested in growth competition experiments (de Ronde et al., 2001; García-Lerma et al., 2001; Yerly et al., 1998). However, those studies included only viruses with 3 or less NRTI resistance-conferring mutations, and none of them contained the i69SS (de Ronde et al., 2001; García-Lerma et al., 2001; Lukashov et al., 2001; Yerly et al., 1998). In contrast, our recombinant viruses had a MNR genotype that included the i69SS and at least 10 RT inhibitor-associated resistance mutations. It has also been reported that, *in vivo*, there is a competitive disadvantage of the insertion mutant compared to the WT virus when therapy is withdrawn (Lukashov et al., 2001). However, the presence of the insertion was always linked to the presence of Tyr at position 215, making difficult to elucidate whether the insertion, or the change at position 215 were responsible for the loss of fitness of the HIV-1 isolates. In addition, removing the dipeptide insertion from a MNR HIV-1 isolate did further reduce its viral fitness (Quiñones-Mateu et al., 2002). This would suggest that the insertion by itself has a limited effect in replication capacity. In contrast, Asn-215, Ser-215 or Thr-215 appear to increase viral fitness and reduce AZT and D4T resistance in different sequence backgrounds. To our knowledge, there is only one report showing co-infection with a HIV isolate containing the 69 insertion and a wild-type HIV strain (Daar et al., 2002). This low transmission rate could be due in part to the low prevalence of the insertion in HIV-treated patients, but also to a potentially poor transmission of these variants because of their low viral fitness. Therefore, the *in vivo* influence of the

reversion from Tyr-215 to Asn, Ser or Thr in such MNR context is unknown, but their clinical relevance cannot be ruled out.

The presence of the 6-bp insertion between codons 69/70 allowed us to obtain length polymorphism results from growth competition experiments involving two HIV-1 strains, one of them containing the insertion. This technique rendered a high throughput and a dynamic quantification range easily detecting down to 10% of any of the viral variants. Sensitivity was 2- to 3-fold higher than using relative peak heights in electropherograms obtained by automated sequencing. In order to verify that the outcompeting strain was the WT HIV-1 and not a fitter recombinant virus lacking the 6-bp insertion, we obtained population based sequences at the end of all growth dual-competition cultures. All sequences showed that, in agreement with the length polymorphism results, the dominant virus was the WT at day 28. Similarly, RT sequencing revealed that individual infectious molecular clones used in replication kinetics based on levels of p24 antigen had a stable genotype over the time of the culture.

In this study we have used recombinant viruses whose replication kinetics were quite similar. In this setting, the competitive-culture approach was more efficient than monitoring viral growth through detection of p24 antigen, in discriminating small differences between viral strains (Martinez-Picado et al., 1999; Prado et al., 2002; Quiñones-Mateu et al., 2002). Differences in relative fitness between the two competing recombinant HIV strains were more clearly observed when dual infections were performed at lower MOIs. Thus, while marginal or no viral population changes were observed over time at an MOI of 0.01, the differences were relevant at a 10-fold lower infectivity (MOI of 0.001). Although such effect had never been described for HIV-1, a similar phenomenon has been observed when two closely related subpopulation of foot-and-mouth disease virus (FMDV) were subjected to growth-competition experiments in BHK-21 cells (Sevilla et al., 1998). One of the populations was found to have a selective advantage over the other only when the competition passages were carried out at low MOI.

Conversely, populations coexisted during serial passages carried out at high MOI, suggesting that small differences in the interaction of the virus with the host cell may contribute to a MOI-dependent selective advantage of one viral subpopulation over a closely related subpopulation (Sevilla et al., 1998). In our HIV-1 growth-competition experiments, p24 antigen production was saturated after 3 days of culture at higher MOI, however it remained closer to an exponential phase over the 7-days culture at lower MOI (Fig. 2A). This might indicate that at an MOI of 0.001 there was a higher number of viral replication cycles allowing the two viral strains to reach a higher level of competition. Although speculative, another molecular mechanism underlying MOI-dependent fitness might rely to functional complementation between the two viruses at high MOI. Thus, assuming that infection of susceptible cells by more than one virus happens more frequently at higher MOI, WT RT might complement the activity of a slightly less processive mutant RT.

In conclusion, the RT residue 215 plays a critical role in both drug resistance and replication capacity of MNR viruses containing the i69SS. Thus, the reversion of Tyr-215 to Thr, Ser or Asn in the sequence context of an MNR virus is sufficient to recover full susceptibility to AZT and D4T, as well as to increase replicative capacity in absence of drug. Moreover, Tyr-215 and i69SS were enough to reduce viral fitness in the absence of high levels of phenotypic resistance to NRTIs regardless of the presence of other NRTI-associated mutations. Additionally, we have demonstrated here that fitness differences of two competing recombinant HIV are MOI-dependent.

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# CAPÍTULO IV

## HIV-1 Fitness Evolution in Antirretroviral Experienced Patients with Sustained CD4+ T Cells but Persistent Virologic Failure

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**Clinical Infectious Diseases, aceptado 2005**

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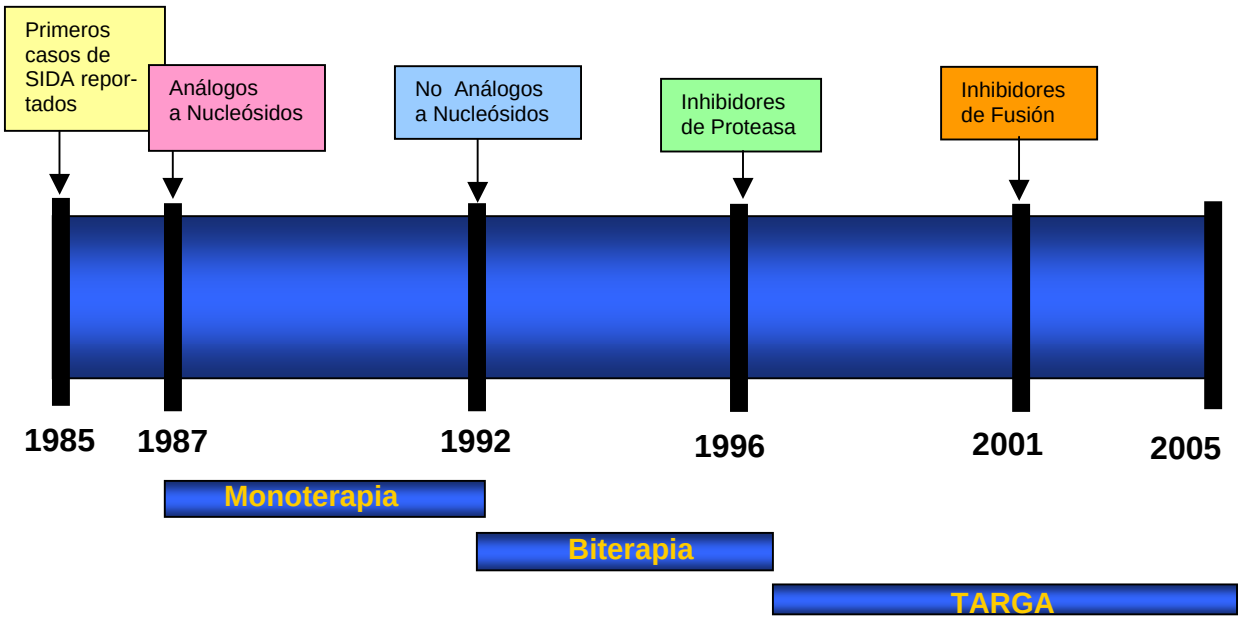



**Cronograma de las estrategias terapeúticas en la historia de la infección por el VIH-1.**

A lo largo de los últimos años los tratamientos antirretrovirales contra el VIH-1 han evolucionado desde mono-terapias a terapias de combinación, en las cuales se incluyen tres o más fármacos activos (TARGA), disminuyendo la tasa de mortalidad en los pacientes infectados por el virus. El trabajo presentado en este capítulo evalúa cambios de "fitness" asociados a la introducción de las diferentes terapias en un grupo de cuatro pacientes con recuento estable de linfocitos CD4 pese al mantenimiento de una carga viral detectable. La evaluación del "fitness" se basa en la construcción de virus recombinantes en pre-terapia y durante el TARGA, conteniendo el extremo 3´terminal de gag y las regiones de la PR y la TI. Los virus seleccionados durante una terapia antirretroviral prolongada resultaron tener un "fitness" menor que los virus seleccionados en fases previas a la terapia o durante los periodos de mono-terapia, con la excepción de uno de los pacientes donde se observó el efecto contrario, coincidiendo con una subida de la carga viral. Además, en este trabajo decidimos evaluar algunos métodos rápidos para la medida del "fitness" que se usan en la actualidad encontrando una buena correlación entre ellos.

## ABSTRACT

***Background:*** Over the last years, treatment guidelines for HIV-infection have evolved from monotherapy to combination regimens including three or more active drugs, which have resulted in a sharp decrease in morbidity and mortality. In the present work, we evaluated changes in HIV-1 viral fitness associated with the sequential introduction of antiretroviral treatment strategies in four chronically infected patients with sustained CD4 cell count despite persistently detectable viral load.

***Methods:*** Pre-treatment and during treatment plasma samples were used to construct recombinant virus containing the 3´end of *gag*, the protease (PR) and the reverse transcriptase (RT) coding region. Drug susceptibility phenotype was evaluated to a panel of multiple RT and PR inhibitors. Replicative Capacity (RC) and infectivity were measured and production of p24 was monitored after transfection.

***Results:*** Multiple Drug-Resistant (MDR) viruses selected during long-term antiretroviral therapy were less fit and infectious than their wild type or monotherapy selected counterparts with the exception of patient B. In three out of four cases p24 kinetics after transfection showed a delay in viral production of recombinant viruses containing MDR mutations. Data from the RC and infectivity assays showed good correlation ($p<0.03$) and corroborated the p24 kinetics data.

***Conclusions:*** This study shows that accumulation of MDR mutations during long-term antiretroviral treatment results, albeit not in all cases, in reductions of viral fitness.

## INTRODUCTION

Since 1987, treatment guidelines for HIV-infection have evolved from monotherapy to combination regimens including three or more active drugs. Nucleoside reverse transcriptase (RT) inhibitors, protease (PR) inhibitors, non-nucleoside RT inhibitors and entry inhibitors have been sequentially introduced in the antirretroviral therapy. The incorporation of these combination regimens have contributed to decrease mortality and morbidity among HIV-1 infected patients [1, 2]. In the course of treatment, however, drug resistant HIV-1 variants often emerge as a result of impotent regimens, suboptimal adherence, pharmacological hurdles or ineffectively treated compartments, which has been a major factor contributing to treatment failure [3, 4].

The frequent bottlenecks introduced by a new antirretroviral treatment lead to variations in viral fitness and quasiespecies distribution of the viral population. Evolution of drug resistance is often characterized by significant fitness losses, which can be partially overcome by compensatory mutations or other adaptative changes [5-7]. However, the overall impact in fitness of the current antirretroviral therapy is still unknown and controversial.

The fact that viral load during treatment failure often remains partially suppressed below pretherapy levels, and that CD4 cell counts remain stable appears to be due to the selection of HIV-1 variants exhibiting decreased replication capacity (RC) [6, 8]. Some studies have shown that highly resistant viruses are less fit than wild type viruses in absence of drug [9-11]. In this regard, the viral RC could be a marker of continued immunological benefit of the antirretroviral regimen. Patients with lower viral RC have significantly greater CD4 cell counts increase from nadir than those with less impaired viruses. This effect seems to be independent of other common clinical markers including reduced phenotypic susceptibility [12].

The aim of this study was to investigate changes in viral fitness associated with the sequential introduction of different antirretroviral drug classes in four chronically HIV-1 infected patients, characterized by a sustained CD4 cell count despite a detectable viral load during long-term antirretroviral

treatment. Thus, we generated recombinant viruses from plasma samples obtained before or shortly after monotherapy initiation, and also a few years later during HAART, and evaluated longitudinal changes in the RC profile of these viruses.

## METHODS

***Patients and plasma samples.*** We studied retrospective samples from four chronically HIV-1 infected patients labeled A through D (Fig.1). Plasma samples were taken and identified with the last two digits of the year of collection as patient's suffix (Fig. 1 and table 1). These patients had sustained CD4 cell counts (losses never superior to 50 CD4 cells over the study period), despite virologic failure (>50 HIV-1 RNA copies/ml), after many years of multiple successive treatments (Table 1). These criteria were fulfilled by 4 out of 13 patients (31%) in our database

***Construction of gag-pol recombinant virus***. Recombinant virus were constructed as previously reported [13]. Viral RNA was extracted (Qiagen, Barcelona) from plasma samples and RT-PCR amplified (One-step RT-PCR, Qiagen, Barcelona) from position 1,811(in $p7^{gag}$) to 4,359 (in $p31^{pol}$). PCR products were cotransfected with the cloning vector pJM31ΔGPRT for homologous recombination in MT2 cells [13]. Viral production after transfection was measured by means of p24 antigen (Innogenetics, Barcelona) every 3 days. Newly generated viral stocks were sequenced.

***Construction of Multiple Single clones.*** Plasma HIV RNA from patient A was reverse transcribed, PCR-amplified and used to construct multiple clones containing the 3´-end of gag and pol coding region in pIE13 vector. pIE13 vector is a $HIV_{NL43}$ based plasmid that has had removed the fragment from the ApaI site (1988) to the EcoRI site (5743), and substituted for the equivalent fragment derived from pJM13ΔGPRT [13], making a $HIV_{NL43}$ plasmid that lacks the 3'-end of gag, the PR and the RT. Ten molecular clones obtained during monotherapy with AZT ($A_{93}$) and ten clones obtained during HAART ($A_{00}$) were transfected by electroporation in MT2 cells. Viral production after transfection of each individual clone was monitored by p24 antigen in the culture supernatants.

***Genotyping***. Plasma samples and their correspondent *pol* recombinant viral stocks were sequenced to confirm the presence in the viral stocks of the same viral variant sequenced in plasma as the main viral replicating population. Individual molecular clones from patient A were sequenced to con-

firm the presence of the insert and to evaluate the quasispecies variation. Phylogenetic analysis confirmed that all samples from the same patient clustered together and excluded cross-contamination among recombinant viruses.

***Drug susceptibility assay.*** Testing of drug susceptibility was performed on frozen culture supernatants of recombinant virus, by means of a rapid recombinant virus assay (PhenoSense HIV, ViroLogic) [14]. This assay involves the construction of resistance test vectors, which are comprised of a pool of recombinant HIV-1 containing gag (3'-end from p7), PR and RT sequences derived from the virus sample that is being evaluated. Resistance test vectors also contain a luciferase reporter gene replacing *env* to monitor a single round of virus replication. Susceptibility of resistance test vectors to a panel of antiretroviral drugs was compared to a reference vector containing the PR and RT sequences derived from HIV-$1_{NL4-3}$. Two independent measurements of each viral isolate were obtained.

***Replication Capacity (RC) assay..*** RC was measured using a modified version of the PhenoSense drug-susceptibility assay [15, 16].The relative replication efficiencies of the isolates was also measured as a function of the level of p24 antigen in culture supernatants [17]. The replication constant (K) was measured as the time in days to reach 100,000 pg/ml of p24 antigen after transfection. The relative replication efficiency of the isolates was determined from the ratios of K of recombinant viruses ($K_{rv}$) from the patients over K for a wild type strain HIV-$1_{NL43}$ ($K_{wt}$) and multiplied by 100.

***Single cycle infectivity assay. .*** Infectivity of viral stocks was measured by the single-cycle infectivity assay in Ghost-CXCR4 cells as previously described [18] with modifications. A total of 5 X $10^4$ cells/well were infected in triplicate with 50 ng of p24 antigen equivalent of virus in the presence of 20 μg of Polybrene/ml by spinoculation (3h at 1,500 X g and 22ºC). Proportion of GFP positive cells was measured by fluorescence-activated cell sorting (FACS) analysis 24h post-infection.Infectivity.

***Statistical analyses*.** Statistical analyses were performed using the Graph-Pad Prism version 4 (GraphPad Software, San Diego, CA, USA). The correlation analyses were estimated using Pearson correlation test. All *P* values were two-tailed.

## RESULTS

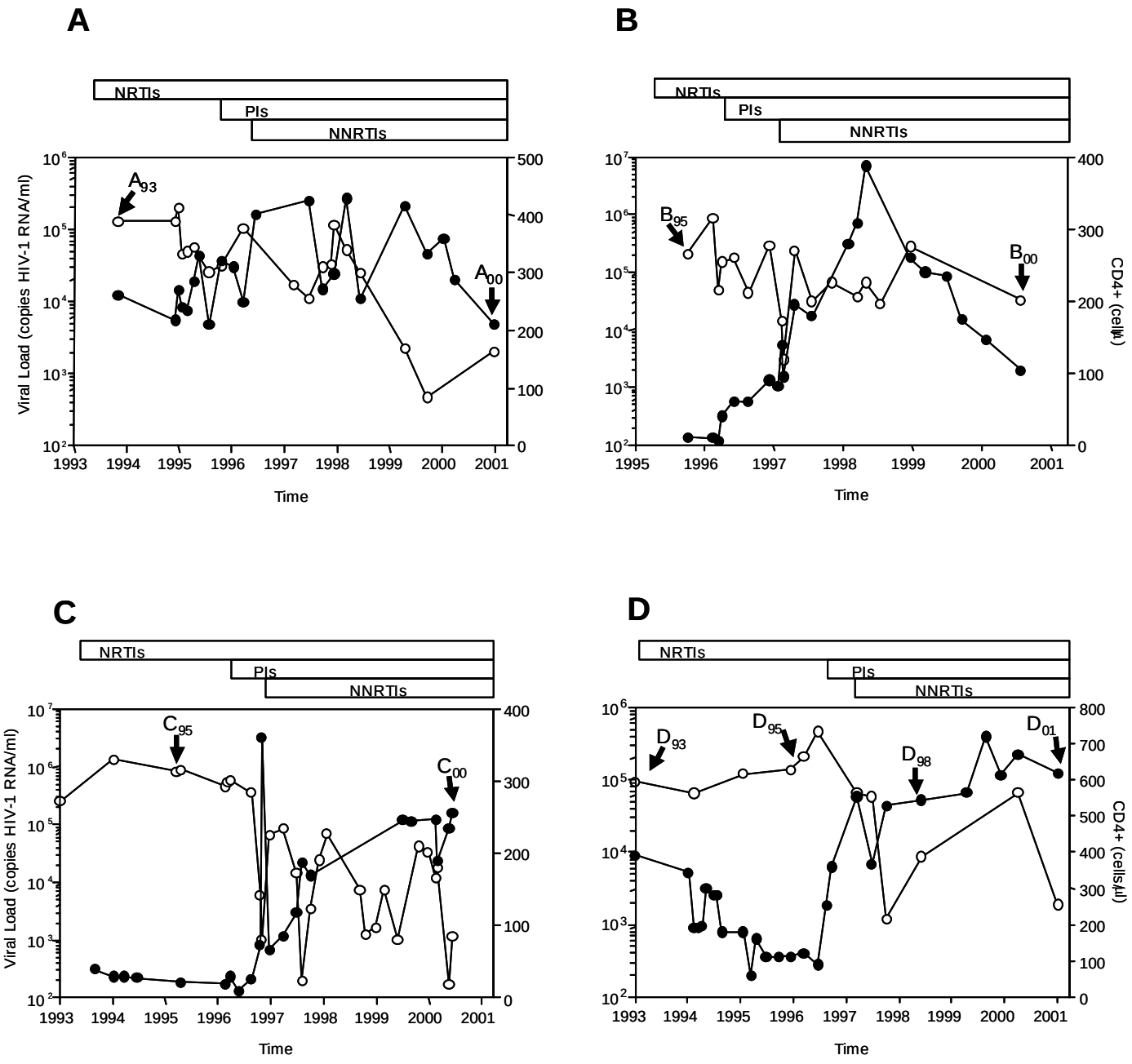
### *Clinical parameters.*

The median clinical follow-up of the patients in the study was of 8 years (IQR: 6.5 - 8.0) (Fig. 1). Plasma viral load (pVL) in these patients dropped a median of 1.7 log HIV-1 RNA copies/ml between the beginning and the end of the study period (IQR: 1.2 - 1.8), but never reached less than 50 copies despite multiple switches of therapy pursuing that goal. Nevertheless, they had a median CD4 gain of 24 cells/year although this increment was not constant during the study period. These patients initiated AZT monotherapy in the first half of the nineties, then were switched to dual therapy and finally to HAART according to the HIV-1 treatment guidelines upgrades. All four subjects were initially treated with NRTIs, they had PIs introduced in their antiretroviral regimens between 96 and 97, mostly associated with significant increments in CD4 cell counts and reductions in pVL, and later on they added NNRTIs to their treatments. At the time of the last sampling they had received a median of 10 (range: 9 - 13) antiretroviral drugs including those from the last therapeutic regimen.

### *Genotypic and phenotypic analysis.*

Genotype of the patients was analyzed at different time points (Table 1). Patients at pre-treatment or monotherapy ($A_{93}$, $B_{95}$, $C_{95}$ and $D_{93}$) carried a median of 2.5 resistance mutations in the RT and no resistance mutations in the PR. After successive switches of antiretroviral treatment, at the last sampling point ($A_{00}$, $B_{00}$, $C_{00}$ and $D_{01}$) there was an increase in the median number of primary resistance mutations [19] to 7.3 for the RT and to 3.5 for the PR. Phenotypic analyses was in accordance with the genotypic results. Thus, antiretroviral drug susceptibility decreased as the number of drug resistance mutations increased. Pretherapy and monotherapy recombinant viruses remained susceptible to all antiretroviral drugs with the exception of AZT. However, later viruses were fully resistant to all NNRTIs, all PIs and most of the NRTIs assayed. The sample $D_{95}$, taken at the time of triple therapy exclusively with NRTIs, was phenotipically cross resistant to differ-

ent NRTIs but retained susceptibility to PIs and NNRTIs in accordance to the plasma genotype.




**(Fig.1).Schematic representations of patients (A to D) included in the study**. CD4 counts are represented with close dot symbols and plasma viral load with white dot symbols. Boxes in the upper part of the figure show introduction of different drug classes. Arrows indicate sampling points.

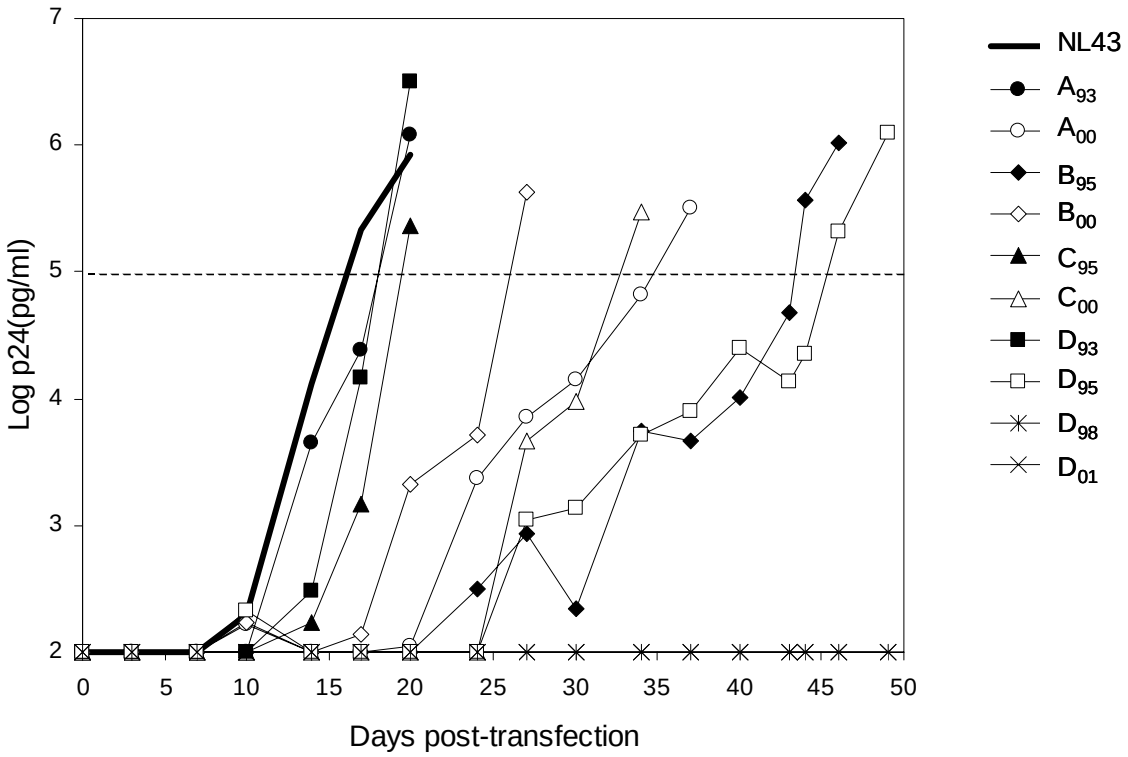
**Table 1.** Patients' characteristics during the study follow-up including amino acid substitutions in PR and RT

| Sample ID[1] | Year | pVL[2] (HIV RNA copies/ml) | CD4 (cell/µl) | ART[3] | Primary Resistance Mutations | |
|---|---|---|---|---|---|---|
| | | | | | PR | RT |
| $A_{93}$ | 1993 | 128,455 | 261 | AZT | wt[4] | D67N;K70R |
| $A_{00}$ | 2000 | 2,000 | 211 | ddI+ABV+TDF+SQV+LPV/r | M46I;I54V;I84V;L90M | M41L;D67N;K70R;K103N; Y181C;T215F;K219Q |
| $B_{95}$ | 1995 | 209,428 | 10 | Treatment naïve | wt | wt |
| $B_{00}$ | 2000 | 33,000 | 104 | RTV+SQV+ABV+3TC | G48V;I54V;V82A;L90M | M41L;L74V;V108I;Y115F;G190A;Y181C; M184V;T215Y |
| $C_{95}$ | 1995 | 827,635 | 22 | AZT | wt | M41L;L210W;T215Y |
| $C_{00}$ | 2000 | 18,211 | 246 | NVP+NFV+SQV+ABV+3TC | M46L;I54V;V82A | M41L;L74V;V118I;Y181C; M184V;L210W;T215Y |
| $D_{93}$ | 1993 | 93,310 | 391 | AZT | wt | D67N;T69N;K70R; T215F;K219Q |
| $D_{95}$ | 1995 | 137,926 | 111 | AZT+ddC+3TC | wt | D67N;T69D;K70R ;M184V T215F;K219Q |
| $D_{98}$ | 1998 | 8,680 | 544 | d4T+RTV+SQV+3TC | I54V;V82A;I84V;L90M | M41L;D67N;T69D;K70R;M184V T215F;K219Q |
| $D_{01}$ | 2001 | 1,900 | 619 | RTV+NFV+SQV+3TC | I54V;I84V;L90M | D67N;T69D;K70R;K103N ;V108I M184V; K219Q |

[1] The letter identifies the patient and the number identifies the year of sampling; [2] plasma viral load; [3] antirretroviral treatment. [4] wild-type. During the total follow-up period patient A received AZT, 3TC, ddI, ddC, d4T, ABV, TDF, INV, RTV, SQV, NVP, NFV, LPV/r; patient B received 3TC, ddI, d4T, ABV,

### *Kinetics of p24 after transfection.*

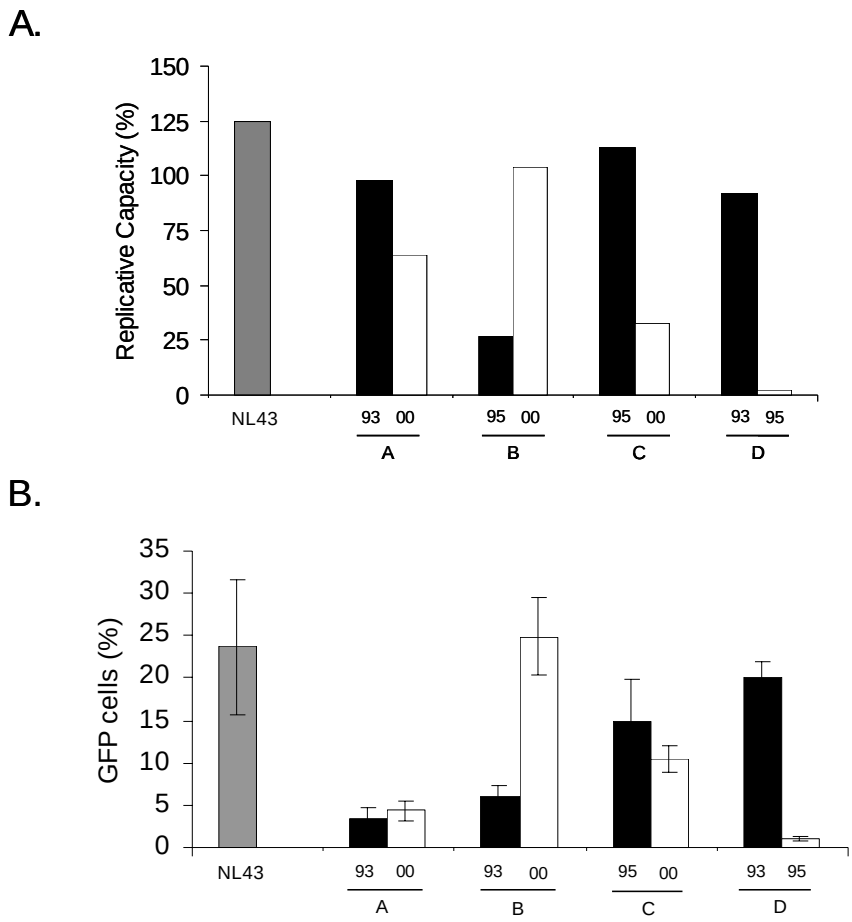
Kinetics of p24 antigen production after transfection showed faster viral replication for recombinant viruses $A_{93}$, $C_{95}$ and $D_{93}$ than for multi drug-resistant (MDR) viruses (Fig.2). Recombinant viruses $A_{93}$, $C_{95}$ and $D_{93}$ produced more than 100,000 pg/ml at day 20 after transfection, similar to the HIV-$1_{NL4-3}$, while their MDR counterparts ($A_{00}$, $C_{00}$ and $D_{95}$-$D_{01}$) produced 100 times less. Conversely, MDR viruses from patient B ($B_{00}$) produced higher levels of p24 antigen after transfection than their wild type counterpart ($B_{95}$). Two of the recombinants viruses for patient D ($D_{98}$ and $D_{01}$) did not replicate enough to obtain viral stocks that allow further experiments.



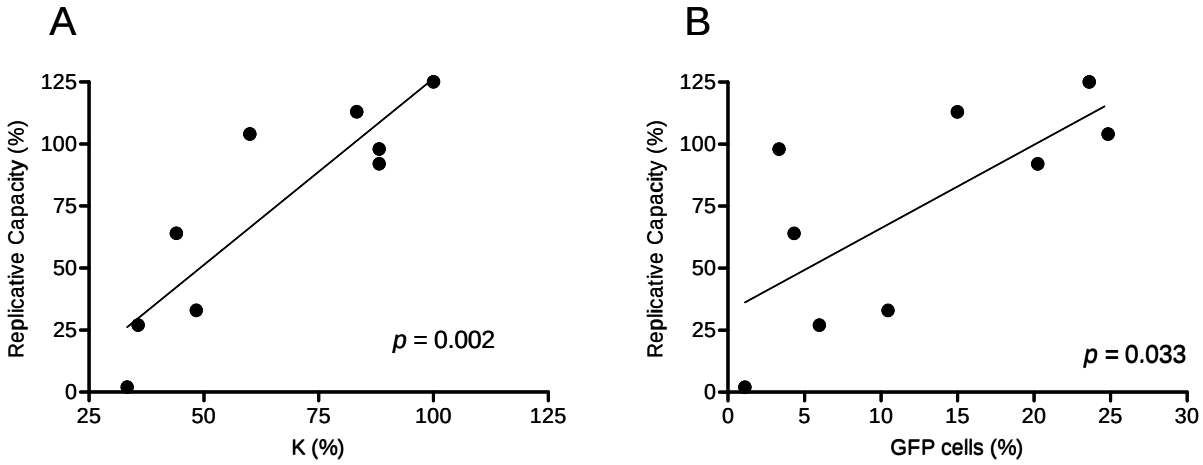

**(Fig.2) Replication kinetics after transfection of MT2 with recombinant virus.** Viral replication was measured by p24 antigen level in cell culture supernatants every three days after transfection. Dashed line indicates the $10^5$ (pg of p24 per ml of culture supernatant). Data were used as a replication parameter to calculate the replication constant (K).

### *Replication Capacity assays.*

We used two independent viral fitness measurements: the single-cycle replication assay from ViroLogic and the infectivity assay in Ghost-X4 cells. Similar results were obtained with both assays (Fig 3). Recombinant viruses obtained during pre-therapy or monotherapy periods had higher RC and infectivity, than the later recombinant MDR viruses, with the exception of patient B for whom opposite results were observed consistently in both assays. Of note, the $A_{93}$ recombinant virus showed better RC than its correspondent MDR $A_{00}$ in the single-cycle replication assay but the infectivity of both viruses was similar. Significative correlation was found between both assays (p=0.033; Fig. 4A), as well as between the RC single-cycle assay and the replication constant (K) (p=0.002; Fig. 4B).




**(Fig.3) Replication capacity and infectivity assay**. Gray bars represent viruses pre-treatment or monotherapy and white bars represent later viruses. All results are expressed in percentages compared to the HIV-$1_{NL43}$. A) Single cycle RC assay measured with a modification of the PhenoSense assay; B) infectivity measured in Ghost-X4 cell line.




**(Fig 4) Correlation between fitness assays. A)** Correlation between RC and replication efficiency constant (K); **B)** correlation between RC and infectivity.

### *Single clone transfection in patient A.*

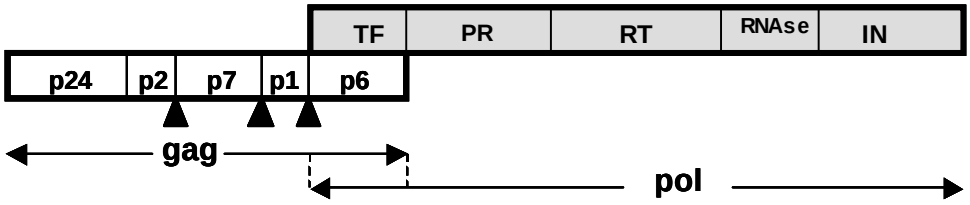
Different viral clones from patient A were constructed containing the 3´-end of *gag* and *-pol* coding regions in the pIE13 vector. Individual clones were transfected. Four out of nine (44%) molecular clones obtained during AZT monotherapy ($A_{93}$) produced p24 antigen while only two out of eight (25%) of the clones containing MDR mutations to all drug classes ($A_{00}$) rendered detectable p24 antigen. Clones transfected were sequenced to observe if those clones that did not replicate have any genotypic difference in comparison to those producing detectable levels of p24. Clone $A_{93}$-10 carried a stop codon at position 24 of the RT, and clone $A_{00}$-06 had the substitution N88S in PR, which has been associated with severe defects in viral infectivity and fitness [20].

### *Cleavage site mutations and non-cleavage site mutations in gag.*

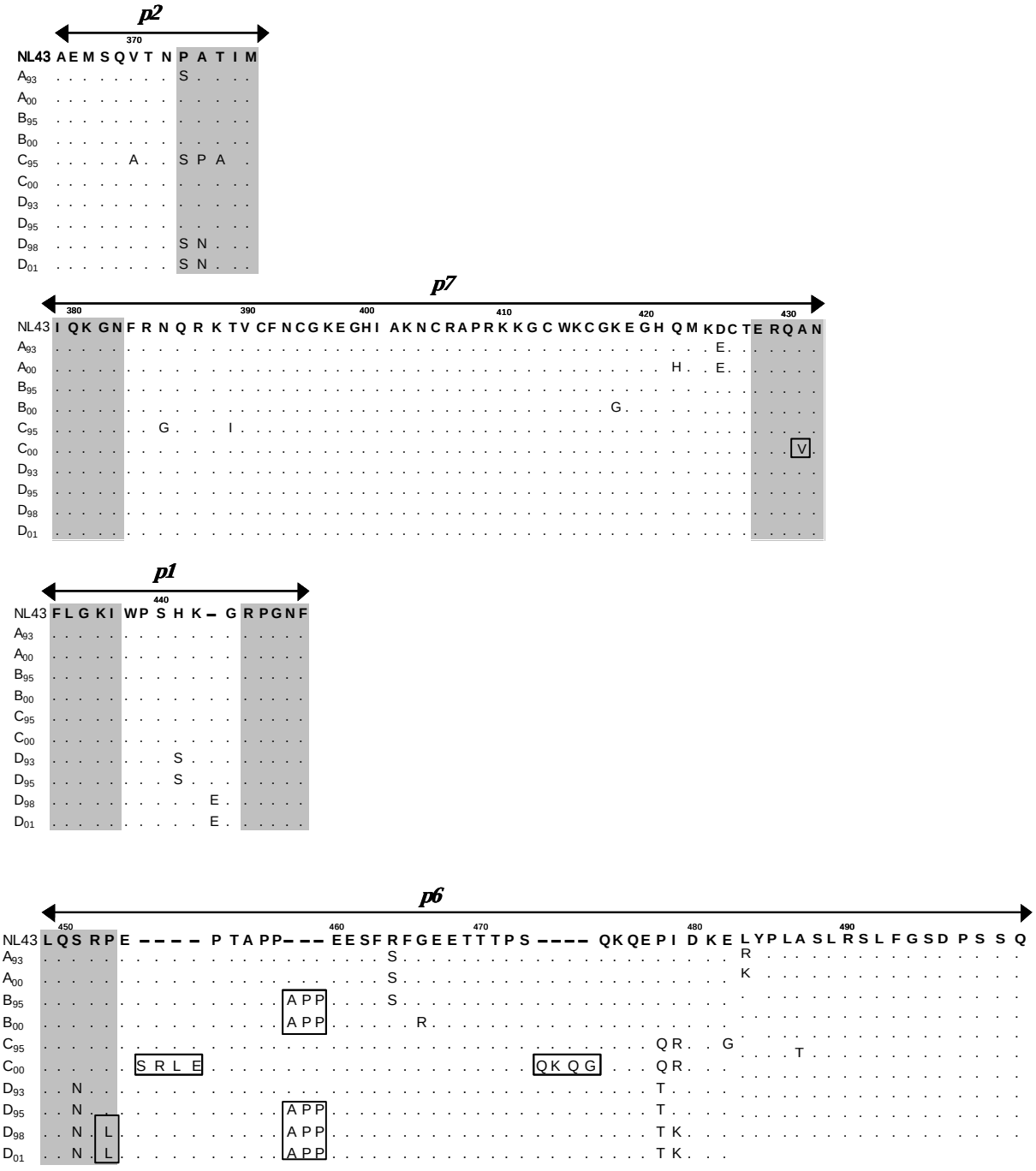
The accumulation of mutations in the *gag* gene has been associated with a recovering of viral fitness in PR inhibitor resistant virus. We sequenced the *gag*-p2 to p6 fragment of the recombinant virus obtained in order to evaluate the emergence of cleavage site mutations (CSM) and non-cleavage site mutations (non-CSM) during antiretroviral therapy (Fig. 5A). There was a

high variation of *gag* mutations for MDR viruses in comparison to their correspondent earlier viruses. Individually taken, antiretroviral treatment selected mutations A431V at the p7/p1 cleavage site developed in patient C (Fig. 5B), along with M46L and V82A in the protease (Table 1). P453L at the p1/p6 cleavage site developed in patient D, concomitantly to the selection of PR I84V. Finally, the introduction of protease inhibitors in patient D selected mutations P373S and A374N at the p2/p7 cleavage. In this analysis we found that 60% (6/10) of the viruses sequenced contained previously reported insertions in p6. Five viruses carried a duplication of three amino acids (APP) in the p6 late motif ($B_{95}$, $B_{00}$, $D_{95}$, $D_{98}$, $D_{01}$). Patient D selected also a one amino acid (E) insertion in position 442 of p2. The sixth virus ($C_{00}$) selected two insertions in p6 over treatment: one at position 454 (SRLE) and the other (QKQG) contiguous to the KQE motif. There is no apparent association between these insertions and treatment, although numbers are small.

a.




b.




**(Fig. 5). Cleavage site and non-cleavage site mutations**. A) Schematic representation of *gag* and *gag-pol* genes. Arrows below figure indicate *gag* and *gag-pol* genes, dashed lines between arrows show frame shift area for gag and gag-pol. Black triangles show cleavage sites (p2/p7, p7/p1 and p1/p6). CA, capsid; TF, trans frame; PR, protease; RT, reverse transcriptase; IN, integrase. B) *gag* sequences of recombinant virus. Alignment was done according to HIV-$1_{NL43}$ reference sequence. Cleavage sites for HIV-$1_{NL43}$ sequence consensus are gray filled. Boxes indicate variations in cleavage sites and non-cleavage with regard to HIV-$1_{NL43}$ previously reported.

## DISCUSSION

In the present study we have used different methodologies to evaluated fitness of recombinant viruses present before therapy or selected during monotherapy and their counterparts selected during highly active antiretroviral regimens (HAART) in patients with sustained CD4+ cell counts but persistent virologic failure.

The relationship between viral fitness and clinical parameters in HIV-1 infected individuals is complex. The presence of MDR viruses with impaired fitness has been associated with sustained CD4 cell counts despite detectable plasma viraemia, suggesting an immunological benefit for the patient [15, 21]. The fitness values of the recombinant viruses used in our study could have contributed to the stability of the CD4 cell counts over the eight years of the study. Presumably, the lost of CD4 cells in patient B during the last two years of follow up after an initially sharp increase might be related to the improved fitness of the recombinant virus $B_{00}$.

Currently, the evolution of HIV-1 fitness in antiretroviral-experienced patients cannot be predicted based on the genotype of drug-targeted genes. The MDR viruses with reduced drug susceptibility that were selected during HAART from patients A, C and D had lower RC and infectivity than their corresponding AZT-resistant recombinant viruses. For patient D, the selection of the substitution M184V in the RT resulted in a severe fitness loss [22, 23]. Further accumulation of new mutations as treatment progressed made impossible to obtain a viral stock for in vitro fitness evaluation, despite persistent plasma viraemia. In patient B, however, the RC of the pretreatment recombinant virus ($B_{95}$) was significantly impaired (27%; the 44% would correspond to the 10th percentile of the wild-type population, median RC = 100%) [24]. Although it is in general accepted that drug resistance mutations emerge at the expense of a loss in viral fitness, the RC of virus $B_{00}$ increased up to 104%, despite progressive accumulation of drug resistant mutations. However, we cannot rule out the effect of non drug resistance associated mutations in RC changes. Therefore, intrapatient comparisons

are far more interesting, as shown here, than the relative RC vs. viral reference strains to assess viral fitness evolution during antiretroviral treatment.

The discrepancy between patient B's pair viruses and the rest of patients was consistent among the different methods used in the study. Indeed, data obtained with the single-cycle assay significantly correlated with replication constant and infectivity results. Because there are many factors that are relevant in the development of appropriate comparative fitness assays, consistency among the different type of fitness experiments employed is important. Because we exclusively used chimeric viruses, we cannot exclude that MDR isolates had altered infectivity and growth kinetics compared to drug-susceptible viruses as shown in the context of primary HIV-1 infection [25].

The accumulation of mutations might result in not only highly crippled but also non-viable viruses. Thus, in the case of patient A we observed a reduction in the efficiency of single-clone transfection concomitantly to the RC impairment. Some changes in the clones transfected could be linked to genotypic determinants associated to severe defects in infectivity and fitness, for example the presence of N88S mutation in the PR gene [20], or the presence of a stop codon in the RT gene, either as a result of viral polymerases or PCR *in vitro* based polymerases. However, most of the clonal sequences had a complex mutational pattern in the PR and the RT making very difficult to associate the lack of virus growing with specific amino acid changes. The accumulation of mutations during treatment failure might result in fewer viable viral clones after transfection suggesting a diminution of the effective viral population after many years of treatment. This reduction of viral viability might also be associated with an increase of the mutagenesis rate through continuous drug pressure reducing fitness, infectivity and contributing to a reduction in the CD4 infected pool. Reductions of the effective viral population due to drug pressure has been reported before for HIV-1 and foot-and-mouth disease viruses [26, 27].

Although the patterns of mutations involved in RC changes are not clearly defined, it has been proposed an important role of *pol* and *gag* coevolution in patients receiving antiretroviral therapy [28-33]. Selection of mutations in *gag* (both CSM and non-CSM) has been previously described as a way to improve viral fitness in highly resistant viruses [25, 31-35]. The CSM A431V has been associated with PR inhibitor resistance mutations at codons 46 and 82 [30, 36], while P453L might direct the PR resistance pathway through I84V instead of V82A mutation [36] in agreement with the emergence of these CSM in patients C and D. The 3-amino-acid duplication (APP) in the proline-rich $p6^{Gag}$ PTAP motif, which interacts with cellular proteins crucial for viral budding, has been linked to an increased infectivity and resistance to NRTIs [28], which is consistent with its emergence before the introduction of PR inhibitors in patients B and D. This insertion, as well as an insertion similar to the SRLE developed in patient C have been recently associated to restoration of reduced RC in MDR viruses [37]. While the presence of the APP insertion in $p6^{Gag}$ in the recombinant viruses from patient B might be related to the increase in RC observed in the MDR virus ($B_{00}$) respect to the pre-treatment virus ($B_{95}$), the emergence of the same insertion in the recombinant virus $D_{98}$, did not seem to compensate for the selection of five thymidine-associated mutations and the RT M184V. MDR recombinant viruses from patients A, C and D did not recover RC to similar or higher levels than earlier viruses despite an accumulation of CSM and non-CSM. By contrast, MDR virus from patient B had an improved RC with respect to the pre-treatment. Only two non-CSM (K418G and G466R) emerged during treatment, but it has not been described whether these mutations might play a role as RC compensatory changes.

Although we focused on the role of gag-pol in RC, overall CD4 and viremia evolution in these patients can be in part consequence of complex virus-host interactions as changes in viral tropism or adaptative immune response, which go beyond the scope of this study.

Our findings challenge the idea that the accumulation of drug-resistance mutations during long-term antiretroviral treatment inevitably results in viruses with impaired fitness. However, MDR viruses with reduced fitness are related to sustained CD4 cell counts in patients with persistently detectable plasma viraemia. Changes in replication capacity and infectivity would be modulated by drug pressure and virus-host interactions playing an important role in the maintenance of the immunologic status. Therefore, the therapeutic benefits of locking into the virus genome drug resistance mutations, which impair viral fitness remains highly speculative.

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# CAPÍTULO V

Discusión y perspectivas

Los capítulos de esta tesis nos ayudan a compreder las consecuencias sujetas a la aparición de mutaciones de resistencia a los fármacos antirretrovirales diseñados para combatir la infección por el VIH-1. La aparición de mutaciones de resistencia en los genes diana (TI y PR) conducen al fracaso de las terapias y a cambios en la capacidad replicativa del virus. A lo largo de los capítulos que componen la tesis, hemos diseccionado el efecto del "fitness" viral en genes individuales como PR (capítulo II) y TI (capítulo III) hasta integrar diferentes genes (capítulo IV). La tesis se ha estructurado en los diferentes niveles de estudio que se detallan a continuación:

- Virus resistentes seleccionados *in vitro* en presencia del inhibidor de proteasa Amprenavir.

- Virus recombinantes multirresistentes a análogos de la TI procedentes de la muestra de plasma de un paciente multitratado.

- Virus recombinantes en un grupo de pacientes con infección crónica. Este grupo de pacientes mantenía un recuento de CD4 estables en presencia de elevados niveles de RNA viral en plasma.

## "FITNESS" Y COMPARACIÓN DE METODOLOGÍAS

En la introducción ya habíamos descrito la importancia que tiene para la medida del "fitness" viral el establecimiento de una metodología de trabajo, y la dificultad que supone la estandarización de la misma [1, 2]. Uno de los objetivos que nos propusimos fue precisamente poder establecer las limitaciones, y utilidades de cada una de las técnicas usadas para cuantificar el "fitness" viral (Tabla.1).

Si bien las técnicas usadas son bastante homogéneas hemos podido observar que no todas tienen las mismas utilidades, ya que no todos los virus presentan los mismos perfiles de "fitness" con la adquisición de mutaciones de resistencia. Para los virus resistentes al Amprenavir la "fitness" se midió por diversas técnicas: cinéticas de replicación, experimentos de un solo ciclo de replicación, análisis de maduración proteica y experimentos de competición. La consistencia de los resultados obtenidos entre los diversos métodos no evita que algunas de las técnicas (como las cinéticas de replicación en cultivos independientes para los virus resistentes a inhibidores de la TI), no permitan discernir cambios de "fitness". En cambio, los experimentos de competición para todos los virus estudiados resultaron ser el método más sensible y definitorio en la medida relativa del "fitness" entre variantes virales, apreciación que ha coincidido con otros autores [3-6]. En este tipo de experimentos la multiplicidad de infección y las dinámicas de los cultivos, medidas por el mantenimiento de la viabilidad celular y una replicación viral activa, son claves a la hora de minimizar la recombinación y maximizar la competición entre las variantes. Un ejemplo de este fenómeno aparece en el capítulo III donde comparamos dos experimentos con diferentes multiplicidades de infección. Sólo el uso de multiplicidades de infección bajas nos permitió establecer una dinámica de competición viral activa, logrando así ver diferencias de "fitness" entre los virus en estudio.

Paralelamente al estudio de los mutantes multirresistentes, iniciamos una colaboración con otro equipo de investigadores para, a nivel bioquímico, evaluar los cambios en la actividad enzimática de la TI de estos mismos mutan-

tes. De esta colaboración surgió una publicación en la cual corroborábamos los datos obtenidos en los experimentos de cultivo celular [7]. La comparación de resultados entre experimentos enzimáticos y experimentos de capacidad replicativa, permite ver cómo defectos en la actividad enzimática de la TI se reflejan en una modificación de la capacidad replicativa de las mismas variantes genotípicas en cultivo.

**(Tabla.1)** Métodos usados en la tesis para la medida del "fitness" viral del VIH-1.

| METODOLOGÍA | UTILIDAD | LIMITACIONES |
|---|---|---|
| Experimentos de un ciclo de replicación | • Rápida (24 ó 48 horas)<br>• Existe una versión comercial<br>• Correlación con datos obtenidos por otros ensayos | • Evaluación de genes individuales<br>• Limitado a un sólo ciclo de replicación<br>• Dificultad para ver pequeñas diferencias de "fitness" entre dos variantes |
| Experimentos de infectividad en Ghost | • Rápida (24 ó 48 horas)<br>• Correlación con los datos obtenidos por los ensayos de un solo ciclo de replicación | • Limitado a un sólo ciclo de replicación<br>• Dificultad para ver pequeñas diferencias de "fitness" entre dos variantes |
| Análisis de maduración de proteínas viales | • Medidas de los defectos de procesamiento de poliproteínas por las PR mutantes | • Semicuantitativo<br>• Posibilidad de compensación del defecto proteico en otro punto del ciclo de replicación |
| Cinéticas de replicación viral | • Medida de la tasa de replicación viral | • Dificultad para ver pequeñas diferencias de "fitness" entre dos variantes<br>• Laborioso |
| Experimentos de competición | • Medida más exacta de "fitness" entre dos variantes virales | • Laborioso por el tiempo del ensayo<br>• Costoso por los métodos de la estimación de cada una de las poblaciones |

Otro punto a destacar ha sido la correlación encontrada en el análisis de diferentes técnicas rápidas para la medida de "fitness" viral. La evaluación del efecto de unos genes concretos y el análisis de los resultados tras un sólo ciclo de replicación son algunas de las limitaciones de este tipo de experimentos.

Uno de los factores que modifican las medidas de "fitness" viral es el tipo de virus usado en los experimentos. Se han descrito variaciones en las medidas de la capacidad replicativa y de la patogenicidad de aislados virales en comparación con virus recombinantes procedentes de muestras de plasma de pacientes [8]. Estas diferencias probablemente se verían aumentadas en aquellos grupos de pacientes que han pasado por múltiples cambios de tratamiento y acumulado mutaciones a lo largo de todo el genoma. La aparición de mutaciones compensatorias en otros genes virales, las alteraciones en el tropismo viral y las interacciones epistáticas entre varios genes complican metodológicamente el estudio de los determinantes genéticos implicados en el "fitness" viral [9-12]. A pesar de todas las restricciones citadas, durante el tratamiento antirretroviral cabría esperar que la presión de selección dominante viniera dada por los fármacos que actúan sobre dianas concretas (PR, TI), y que la modificación de la capacidad replicativa sería principalmente debida a la aparición de mutaciones en los genes diana y, por tanto, biológicamente relevante.

## "FITNESS" Y RESISTENCIA A LOS IPs

La relación existente entre la adquisición de mutaciones de resistencia y una disminución del "fitness" viral se demostró con las mutaciones de resistencia a los IPs [13, 14]. Además de la fuerte asociación entre el elevado grado de resistencia y defectos en "fitness", los virus con resistencia a IPs poseen una gran plasticidad de rutas mutacionales y un elevado grado de resistencia cruzada. Ejemplos de todas estas características aparecen reflejadas en el capítulo II de esta tesis.

Por un lado, los virus en presencia de Amprenavir acumularon progresivamente mutaciones de resistencia en las posiciones 10, 10+84, 10+46-50, 10+46+47+50 y la mutación L449F en p1/p6 de gag, mostrando un aumento del nivel de resistencia y una disminución en su capacidad replicativa. Otra de las características del VIH-1 es su elevada plasticidad y la constante exploración del espacio de secuencias. Esta búsqueda de nuevas rutas mutacionales está condicionada por la presión de selección y favorece la acumulación de mutaciones en unas posiciones y en un orden concreto. Un ejemplo es el cambio poblacional que se produce como consecuencia de un incremento en la concentración de Amprenavir *in vitro*, el virus mayoritario 10+84 es remplazado por otro con mutaciones en las posiciones 10+46+50 más resistente a las nuevas condiciones del medio. La última de las características que vamos a citar de los IPs es el elevado grado de resistencia cruzada [15], cuya consecuencia es una reducción en las opciones terapéuticas de los pacientes a este grupo de antirretrovirales. Nuestro estudio ilustra muy bien este proceso en el que podemos ver como la aparición de ciertas mutaciones de resistencia al Amprenavir producen un incremento en la resistencia al Lopinavir. El incremento de resistencia a este segundo IP condicionaría, en aquellos grupos de pacientes que han sido tratados previamente con Amprenavir, el éxito de la respuesta a un segundo tratamiento con Lopinavir y en menor grado con Ritonavir, como se ha observado posteriormente gracias a ensayos clínicos [16]. En nuestros estudios también hemos incluido con efecto comparativo, los mutantes puntuales I50V y D30N. Estos mutantes fueron obtenidos por técnicas de mutagénesis dirigida y previamente se habían descrito serios defectos en su capacidad replicativa [17]. La sustitución en la posición I50V, localizada en el flap de la PR, compromete la actividad enzimática de este virus [18], siendo suficiente para incrementar mucho el valor de resistencia al Amprenavir, al mismo tiempo que provoca una disminución en la capacidad replicativa. La falta de viabilidad de este mutante y la reducida capacidad replicativa observada en nuestros estudios nos hacen especular que requiere, para su viabilidad, de otras mutaciones compensatorias como la 46 ó 47, encontradas frecuentemente en aislados clínicos [19,20].

## "FITNESS" Y RESISTENCIA A LOS INHIBIDORES DE LA TI

La existencia de virus con mutaciones en la TI y con un "fitness" viral muy disminuido se ha descrito para virus con mutaciones de resistencia en posiciones M184V, L74V o K65R [21-23] de la TI. En contraposición con estos datos son muchas las variantes, incluyendo aquellas con multirresistencias a análogos a nucleósidos, para las cuales no se han observado reducciones tan drásticas de la capacidad replicativa. Estos resultados pueden deberse a diferencias estructurales entre la PR y la TI viral. La estructura enzimática de la TI admitiría un considerable número de variaciones aminoacídicas sin que ello tuviera efecto en su actividad enzimática [24].

Al contrario de lo que ocurre con los virus resistentes a IPs, para los inhibidores de la TI la resistencia cruzada es un proceso más puntual, sobre todo en el caso de los análogos a nucleósidos, dónde se necesita la acumulación de un número elevado de mutaciones para llegar a este estado. La mayor plasticidad, si cabe, en la búsqueda de rutas mutagénicas y la localización de las mutaciones en zonas más distantes del centro activo del enzima [25] bastarían para otorgar esta diferencia.

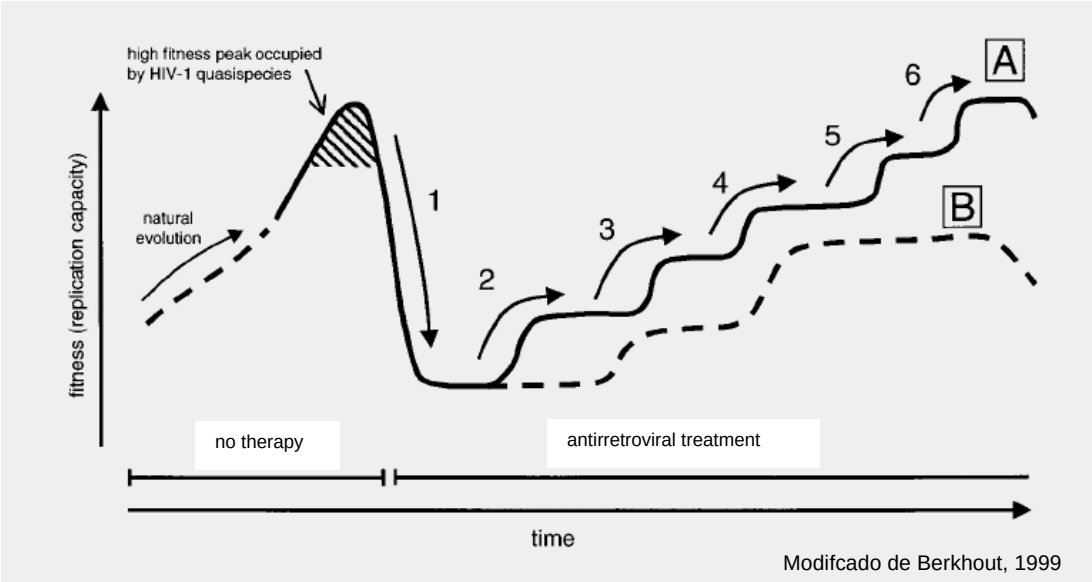
No sólo el "fitness" sino también los perfiles de resistencias de estos mutantes resitentes a inhibidores de la TI juegan un papel importante. A pesar de la acumulación de gran número de mutaciones, la existencia de una serie de mutaciones claves en las que recae el fenómeno de resistencia y en menor grado el de "fitness", nos permite definir cambios aminoacídicos críticos en estos procesos. La reversión de la mutación 215Y a 215N, S o T, que se observa *in vivo* en pacientes que adquieren virus con resistencias al AZT durante la infección primaria [26, 27], es suficiente para recuperar por completo la sensibilidad a los análogos de nucleósido AZT y d4T, al mismo tiempo que observamos una recuperación de la capacidad replicativa.

## "FITNESS" UN PARÁMETRO DINÁMICO

El "fitness" no es una cualidad inherente del virus sino que forma parte de múltiples interacciones virus-huésped donde el virus y el sistema inmunitario pueden actuar como factores moduladores [28-30]. Parámetros como la activación celular de los linfocitos CD8, la actividad específica frente a diferentes epítopos virales, por parte de las células T CD4 [31] como de las células T CD8 citotóxicas [32], o la presencia de anticuerpos neutralizantes frente al virus [33], tienen importancia en la interacción virus-huésped y en la modulación de la actividad viral. El "fitness" es un fenómeno dinámico y asociado a complejas interacciones.

Las cuasiespecies virales están sometidas de forma constante a cambios en el medio y limitaciones del tamaño poblacional. Basándose en las teorías evolutivas de los virus de RNA [34], la evolución natural del VIH-1 sería alterada por la presión de los fármacos antirretrovirales que someten al virus a cuellos de botella, donde el tamaño poblacional y la modulación del "fitness" pasa por diferentes periodos (Fig.1).

El inicio de la terapia antirretroviral favorece el control de la replicación viral durante un periodo más o menos prolongado de tiempo (1 en Fig.1), la falta de efectividad de los tratamientos favorecerá, en estas condiciones, la selección de variantes virales que mantienen su viabilidad en presencia de elevados niveles de fármacos reduciendo su actividad catalítica (2 en Fig.1). La posterior acumulación de mutaciones durante periodos que podríamos denominar de valles y montañas (3-6 en Fig.1) llevaría a recuperar paulatinamente el "fitness" viral a diferentes niveles (A o B en Fig.1). La introducción de nuevos fármacos a lo largo de la terapia antirretroviral y la consiguiente limitación de la replicación conduciría a las cuasiespecies virales a su estado inicial (1 en Fig.1). Sin embargo, los procesos de memoria de las cuasiespecies [35] y la rapidez de emergencia de las variantes resistentes prexistentes, como se ha observado tras la introducción de la terapia en pacientes con virus resistentes sometidos a interrupciones del tratamiento que presentaban cepas salvajes, podrían alterar este patrón.




**(Fig.1) Cambios en la fitness viral antes y después del tratamiento antirretroviral.**

Comprender el "fitness" como un proceso dinámico es indispensable para entender los cambios de capacidad replicativa como mecanismo de adaptación del VIH-1 a las variaciones constantes del medio.

## CAMBIOS DE "FITNESS" DURANTE EL TARGA Y PROGRESIÓN DE LA ENFERMEDAD

La evaluación de los cambios del "fitness" durante el tratamiento antirretroviral es uno de objetivos de esta tesis. Para llevar a cabo este estudio seleccionamos un grupo de cuatro pacientes con infección crónica que mantenían una respuesta inmunitaria, pese a una constante replicación viral. Los antecedentes de nuestro estudio se sitúan en el año 2000, cuando Grabar y col entre otros grupos [36-38] describieron que un 19% de los pacientes que iniciaban TARGA presentaba una respuesta discordante (virológica y/o inmunológica) después de 6 meses de tratamiento. Este estudio evaluó una cohorte de 2236 pacientes no tratados previamente con IPs. Esta respuesta se mantuvo hasta los 12 meses y su riesgo de progresión a SIDA fue similar entre el grupo con sólo respuesta inmunológica y el grupo que respondió completamente al tratamiento. Esta observación llevó posteriormente a varios equipos de investigadores a interesarse por las características inmuno-

lógicas y virológicas que definían a estos individuos [39]. Varios trabajos [30, 40-42] propusieron que una disminución del "fitness" viral de las variantes resistentes seleccionadas favorecería el mantenimiento de las células T en estos pacientes. Los mecanismos por los cuales estos virus replicaban en menor grado y, por lo tanto serían menos patogénicos, se asociaban a la introducción de los IPs. Parecía lógica la relación entre la aparición de mutaciones de resistencia a esta familia de fármacos y una reducción del "fitness", como se había descrito previamente *in vitro*. A posteriori, una publicación planteó que la diferencia de patogenicidad sería debida a una menor mortalidad de las células del timo, en las cuales los virus resistentes a los IPs no podrían replicar con tanta eficiencia como en otros compartimentos celulares [43]. Frente a la multitud de publicaciones surgidas en los últimos años acerca de los fenómenos de "fitness" viral, esta atractiva hipótesis ha quedado relegada a la espera de nuevos estudios que corroboren el papel de las mutaciones de resistencia en la patogénesis de las células T.

Actualmente, se han seguido cohortes de pacientes que mantienen estas características discordantes durante periodos prolongados de tiempo [39, 44], lo que apoya *in vivo* el posible beneficio clínico asociado al mantenimiento de cepas virales resistentes. En este mismo contexto, el estudio presentado en el capítulo IV demuestra que efectivamente en estos pacientes se produce una selección de virus con baja "fitness". Asimismo y en contraste con otros estudios, en uno de los pacientes analizados se aprecia un incremento del "fitness" coincidiendo con una subida de la carga viral y pese a la presencia de múltiples mutaciones de resistencia. Esta observación nos ayuda a matizar la idea de que no siempre una elevada resistencia debe ser sinónimo de un bajo "fitness" y que la variación genotípica del VIH-1 sobrepasa la capacidad de predicción del "fitness" sobre la base del genotipo.

El VIH-1 ha desarrollado múltiples mecanismos de evasión a pesar de los graves defectos replicativos a los que conducen la aparición de ciertas mutaciones. Trabajos recientes han demostrado el efecto de la adquisición de mutaciones, tanto en los sitios de procesamiento de la PR como fuera de éstos,

actuando como mutaciones compensatorias y mejorando el "fitness" de algunas variantes virales [9, 45-47]. Un ejemplo se presenta en el capítulo IV donde hemos visto la aparición de múltiples mutaciones durante el TARGA, tanto en los sitios de procesamiento de la PR como fuera de éstos. Otro ejemplo se cita en el capítulo II donde la emergencia de la mutación L449F en gag p2/p6 y la posibilidad de un efecto compensatorio en el "fitness" se ha demostrado en trabajos posteriores [20]. Entre los mecanismos virales para la recuperación del "fitness" destaca la aparición de inserciones y deleciones en gag. La duplicación del motivo PTAPP de p6 está involucrada en un cambio de la pauta de lectura de gag y un incremento en la producción de proteínas [48, 49] que colaboran en el proceso de infección viral. Asimismo, estudios moleculares han descrito la coevolución de gag con algunas mutaciones de resistencia en la PR [50, 51], aunque la asociación directa de cambios en gag y la presencia de mutaciones de resistencia es todavía contradictoria [52, 53].

## FITNESS COMO MARCADOR CLÍNICO

Desde el inicio de los estudios de "fitness" ha estado presente la polémica sobre su uso como marcador clínico. En base a nuestros estudios el uso del "fitness" viral, como parámetro clínico, debería partir de un seguimiento longitudinal de los valores de "fitness" y la posibilidad de asociación a un genotipo concreto. En los primeros capítulos de la tesis hemos demostrado la asociación entre mutaciones puntuales o patrones mutacionales concretos con una pérdida del "fitness" *in vitro;* el panorama en un paciente es mucho más complejo y la variación genotípica del virus excede la capacidad de predicción del "fitness" en base al genotipo.

La presencia de virus con baja "fitness" en pacientes progresores lentos [54], la existencia de virus poco replicativos en pacientes no tratados [30], junto con las observaciones de que algunas mutaciones de resistencia, como M184V en la TI o la mutación D30N en la PR, conferían baja fitness *in vivo* asociándose con una menor progresión y un mejor pronóstico de la enfermedad [55, 56] apoyan el uso del "fitness" como marcador. La realización de

estudios clínicos a gran escala serían necesarios para dotar a este parámetro de un valor pronóstico definitivo. En la actualidad, el uso de la capacidad replicativa como marcador clínico no puede ser único ni definitorio. El valor de la carga viral y la variación en el recuento de células T CD4 continúan siendo los mejores marcadores clínicos para la monitorización de la infección por el VIH-1.

Los valores de "fitness" nos aportarían una información extra, que podría ser útil para el mantenimiento de la terapia en pacientes con respuesta inmunitaria pero replicación continuada [44]. Alternativamente, en pacientes no tratados con virus poco replicativos el retraso del inicio de la terapia reduciría el periodo de tratamiento y la toxicidad asociada al mismo [23, 30].

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# *CONCLUSIONES*

Las conclusiones más destacables extraídas de los diferentes estudios se pueden resumir de la siguiente forma:

1. Los virus seleccionados in vitro en presencia de Amprenavir acumulan de forma progresiva mutaciones en posiciones 10, 10+84, 10+46+50, 10+46+47+50 de la PR. La selección de estas mutaciones produce un incremento en la resistencia al Amprenavir y de un modo paralelo una disminución del "fitness" viral.

2. Las mutaciones de resistencia al Amprenavir conducen a resistencia cruzada con el Lopinavir y en menor grado con el Ritonavir.

3. En el contexto de multirresistencia a análogos a nucleósidos, cambios aminoacídicos en la posición 215 de la TI, son claves para el mantenimiento de la resistencia al AZT y al d4T.

4. La selección de la mutación 215Y también se asocia a una reducción del "fitness" aunque su impacto es limitado.

5. La selección de virus con baja "fitness", durante el tratamiento antirretroviral, favorece el mantenimiento de una respuesta discordante en algunos pacientes con infección crónica.

6. Las técnicas para la medida de "fitness" e infectividad in vitro de virus resistentes presentan resultados comparables, siendo los experimentos de competición la técnica más sensible para determinar pequeñas diferencias de "fitness" entre dos virus.

7. El "fitness" es un parámetro dinámico y dependiente de multitud de factores virales y del huésped.

# ANEXO I

Protocolos de cultivo celular para medidas de "fitness" viral

# Protocolo I

## CINÉTICAS DE REPLICACIÓN EN CULTIVOS INDEPENDIENTES

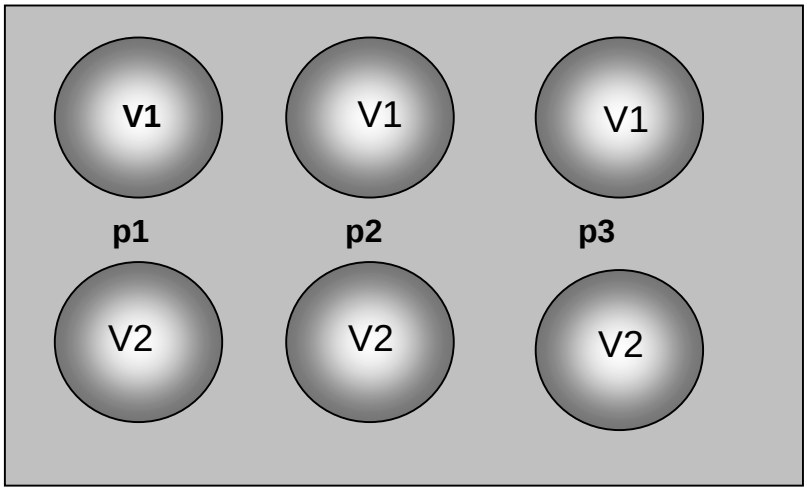
**Materiales necesarios:**

- RPMI 1640 medio (Invitrogene cat.21875091).
- Suero Fetal Bovino (SFB, Sigma cat. F-2442).
- Solución de Penicilina y Streptomicina (Invitrogene cat.150063).
- PHA (Sigma cat.L9132).
- IL-2 (10000U, Roche cat.11204700001).
- PBS 1X (Roche cat.11666789001).
- Placas de 6 pocillos (Afora cat. 3810/006).
- p24 antigen ELISA ( Innogenetics cat.K-1048).
- R10= RPMI 1640+10% SFB+ 100 (µg/ml)Penicilina y Streptomicina.
- R20= RPMI 1640+20% SFB+ 100 (µg/ml)Penicilina y Streptomicina.
- **Células**: líneas celulares linfoides inmortalizadas (MT4, MT2); en caso de usar cultivos primarios, las células se deben estimular 72h antes de la infección con PHA e IL-2 (1%) en R20 (RPMI+ 20% SFB + P/S ).
- **Virus:** Diferentes variantes de VIH-1.

**Método:**

- Hacer un pellet de 7,5 x10 $^{6}$ células para MT2, MT4 y 15 x10 $^{6}$ células para cultivos primarios.
- Infectar con un inóculo de 7500 ó 15000 $TCID_{50}$ para conseguir una MOI de 0.001 (por ejemplo para Multiplicidad de infección [MOI] = $TCID_{50}$/10 $^{6}$células =15000/15 x10 $^{6}$ células = 0.001 hasta un volumen final de 1ml con R10 en un tubo de 13 ml).
- Incubar durante 2h a 37ºC y 5% $CO_2$.
- Lavar el pellet con PBS 1X estéril a 1400 rpm durante 5 min.
- eliminar el sobrenadante y repetir de nuevo el lavado con PBS 1X.
- Resuspender el pellet en 3ml de R10 ó R20 dependiendo del tipo celular usado y añadir 1ml de esta resuspensión en cada pocillo de una placa de 6 haciendo tres réplicas y dejar a una densidad celular de

0,5x10 $^{6}$ (células/ml) en el caso de las líneas celulares MT2 y MT4, y de 1 x10 $^{6}$ (células/ml) para cultivos primarios.




**Placa de 6 pocillos en cultivo por triplicado para cada virus.**

- Recoger alícuotas de 250 µl y añadir la misma cantidad de medio fresco a día 1, 2, 3, 4, 5, 6, 7 y 10.
- Una vez recogidas las muestras se procede a realizar un ELISA con objeto de cuantificar la cantidad de antígeno p24 acumulado en el cultivo a lo largo del tiempo.
- Secuenciar los virus al final de cultivo para asegurarnos de que no se ha producido una reversión del genotipo.
- Para el análisis de datos en logaritmo, al menos 2 réplicas se han de ajustar a un modelo linear por máxima probabilidad. Para cada mutante la tasa de producción de p24 se asume que es la misma entre réplicas, pero la cantidad inicial de p24 puede variar entre réplicas de acuerdo con una distribución normal.

**Consejos:**

- Las diferencias en las densidades celulares finales, dependen del tipo de celular a usar. En el caso de las líneas inmortalizadas su rápido crecimiento limitaría el crecimiento viral por la saturación del cultivo siendo su densidad final de trabajo menor.

Protocolo basado en las siguientes referencias:

Martinez-Picado et al., 1999, J Virol.
Prado et al., 2002, AIDS.
Prado et al., 2004, Virology.

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# Protocolo II

## ENSAYOS DE COMPETICIÓN

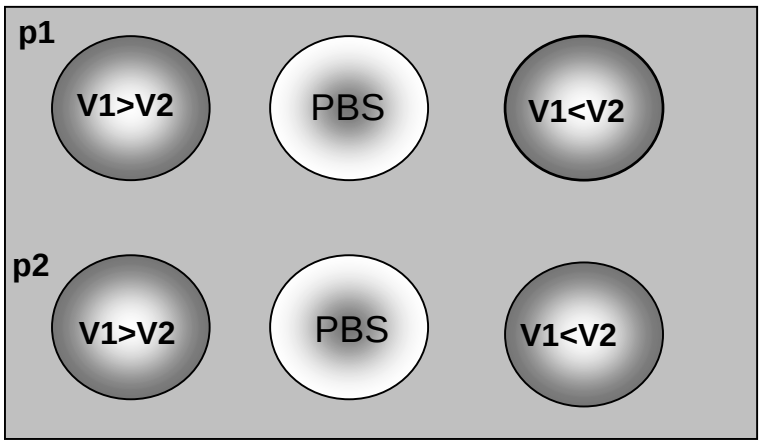
Los ensayos de competición se realizan normalmente en ausencia de fármaco y en líneas celulares linfoides. Como hemos indicado, esta técnica es la más sensible para medir diferencias de "fitness", y se debe de realizar a bajas MOI con el fin de mantener una dinámica de cultivo activa, y evitar los fenómenos de recombinación que podrían dar lugar a nuevas variantes virales diferentes, interfiriendo en los resultados.

### Materiales necesarios:

- RPMI 1640 medio (Invitrogene cat.21875091).
- Suero Fetal Bovino (SFB, Sigma cat. F-2442).
- Solución de Penicilina y Streptomicina (Invitrogene cat.150063).
- PHA (Sigma cat.L9132).
- IL-2 (10000U, Roche cat.11204700001).
- PBS 1X (Roche cat.11666789001).
- Placas de 6 pocillos (Afora cat. 3810/006).
- R10= RPMI 1640+10% SFB+ 100 (µg/ml)Penicilina y Streptomicina.
- R20= RPMI 1640+20% SFB+ 100 (µg/ml)Penicilina y Streptomicina.
- Kit extracción RNAviral (Quiagen cat.52904).
- **Células**: líneas celulares linfoides inmortalizadas (MT4, MT2); en caso de usar cultivos primarios, las células se deben estimular 72h antes de la infección con PHA e IL-2 (1%) en R20 (RPMI+ 20% SFB + P/S ).
- **Virus:** Diferentes variantes de VIH-1.

**Método:**

- Hacer un pellet de 5 x10 $^{6}$ de células para MT2, MT4 y 10 x10 $^{6}$ de células en el caso de cultivos primarios.
- Infectar con un inóculo de 5000 ó 10000 $TCID_{50}$ basándonos en los títulos de infectividad de los diferentes virus, con dos virus (V1 y V2) en diferentes proporciones ( por ejemplo 80% V1> 20% V2 y 20% V1< 80% V2) para conseguir una MOI de 0.001 (por ejemplo para Multiplicidad de infección[MOI] = $TCID_{50}$/10 $^{6}$células = 10000/10 x10 $^{6}$células = 0.001 hasta un volumen final de 1ml con R10 en un tubo de 13 ml).
- Las células también se infectarán por separado con las cepas mutantes y salvajes para evaluar la potencial reversión genética a lo largo del experimento.
- Incubar durante 2h a 37ºC y 5% $CO_2$.
- Lavar el pellet con PBS 1X estéril a 1400 rpm durante 5 min; eliminar el sobrenadante y repetir de nuevo el lavado con PBS 1X.
- Resuspender el pellet en 2ml de R10 ó R20 dependiendo del tipo celular usado.
- Añadir 1ml en cada pocillo a una placa de 6 para hacer dos réplicas y dejar a una densidad celular de 0,5 x10$^{6}$ (células/ml) en el caso de las líneas celulares MT2 y MT4, y de 1 x10$^{6}$ (células/ml) para cultivos primarios.




**Placa de 6 pocillos en cocultivo por duplicado para cada competición.**

- Diluir el cultivo ½ a días 3, 10,17 y recoger muestras.
- Reinfectar 2,5 x10 $^{6}$ células MT2, MT4 ó 5 x$10^{6}$ células con una dilución 1:100 del sobrenadante en 1ml de volumen final a días 7, 14 y 21.
- Incubar durante 2h a 37ºC y 5% $CO_2$.
- Lavar el pellet con PBS 1X estéril a 1400 rpm durante 5 min; eliminar el sobrenadante y repetir de nuevo el lavado con PBS 1X.
- Resuspender el pellet en 1ml de R10 ó R20 dependiendo del tipo celular usado.
- Añadir 1ml en cada pocillo, de la placa de 6 y dejar a una densidad celular de 0,5 x$10^{6}$ (células/ml) en el caso de las líneas celulares MT2 y MT4 y de 1 x$10^{6}$ (células/ml) para cultivos primarios.
- Extraer el RNAviral total a días 3, 7, 10, 14, 17 y 21.
- Secuenciar los cultivos simples a d21 para asegurarse que no hayan ocurrido procesos de reversión durante el cocultivo.
- Analizar los cambios poblacionales mediante la técnica escogida (análisis clonal, técnicas de Genescan, altura de los picos de los electroferogramas).

**Consejos:**

- El tiempo de mantenimiento del cocultivo va a depender de la cantidad de inóculo inicial de cada una de las variantes y de sus diferencias en "fitness". Si en estas condiciones no logramos ver diferencias entre los virus se pueden hacer reinfecciones cada 3 ó 4 días.
- El uso de líneas celulares linfoides favorece la máxima infección de los cultivos, mientras que el uso de cultivos primarios, aunque "más real", se limita a un 5% de las células totales, alargando los periodos de cocultivo si queremos ver diferencias de "fitness" en virus parejos. Además la variabilidad entre donantes en ocasiones puede reducir el crecimiento viral.

Protocolo basado en las siguientes referencias:

Martinez-Picado et al., 1999, J Virol.
Prado et al., 2002, AIDS.
Prado et al., 2004, Virology.

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# Protocolo III

## ENSAYOS DE INFECTIVIDAD EN GHOST

La línea celular Ghost deriva de células humanas de osteosarcoma(HOS), expresan de un modo estable CD4 y los receptores de quimiocinas CXCR4 y CCR5 junto con el gen de la proteína verde fluorescentes (GFP) bajo el control del LTR del HIV-2. Con la entrada del virus y la expresión de la proteína tat unida al promotor de la GFP la fluorescencia producida es un indicador del nivel de infección viral.

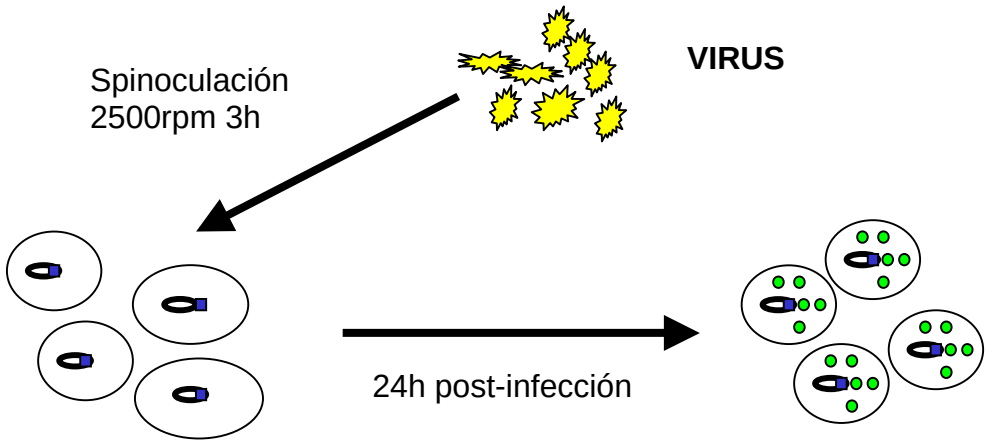
### Materiales necesarios:

- DMEM ( Dulbeco´s Eagle Medio Modificado, Invitrogene cat.31885049).
- Suero Fetal Bovino (SFB, Sigma cat. F-2442).
- -Solución de Penicilina y Streptomicina (Invitrogene cat.150063).
- Geneticina (G418 , Invitrogene cat. 10131019).
- Higromicina (Sigma cat. H3274).
- Puromicina (Sigma cat.P7255).
- Polibreme (Hexadimethride bromide, Sigma cat.H9268).
- Tripsina 2,5%(Invitrogene cat. 250550-014).
- PBS 1X (Roche cat.11666789001).
- Placas de 48 pocillos
- Medio Ghost CXCR4/CCR5= DMEM+10% SFB+ 500 (μg/ml) G418+100(μg/ml) Higromicina+ 100(μg/ml) Penicilina y Streptomicina.
- Formaldehído (Sigma, cat. F1635).
- **Virus:** Diferentes variantes de VIH-1.

### Método:

- El día antes sembrar 50000 (células/ml) en una placa de 48 pocillos.
- La confluencia a las 24 h debe de ser de un 40-50%.

- Retirar el medio y añadir por duplicado la mix de infección: 50ng de sobrenadante de virus hasta un volumen final de 500µl.
- Añadir 5µl de polibreme ( stock 20(mg/ml) diluir 1:10 en agua, guardar el stock a 4ºC).
- Centrifugar 2500rpm durante 3h a 22ºC, este proceso se denomina spinoculación.
- Retirar la mix de infección y añadir 1ml de medio Ghost ( si queremos añadir algún fármaco antirretroviral esperar 3h).
- Dejar 24h a 37ºC y 5% $CO_2$.
- Pasar por el citómetro de flujo las células ( FACS), retirar el sobrenadante de los pocillos, añadir dos gotas de tripsina 2,5% resuspender en 600µl de PBS 1X , dejar unos segundos retirar las células del pocillo a un tubo de FACS.
- Lavar dos veces con PBS 1X a 1400rpm 5min.
- Resuspender en 300µl de formaldehído 2%.




**Representación esquemática del ensayo de Ghost**.

**Consejos:**

- El control negativo es imprescindible para el análisis por FACS ya que existe fluorescencia de fondo y tenemos pocas células.
- A las 24h mirar en el microscopio de fluorescencia si no vemos verde es aconsejable esperar hasta las 48h.

- El polibreme es un polímero catiónico muy tóxico para las células y lábil. Se recomienda conservar a 4ºC a una concentración de 20 mg/ml durante un periodo no superior a 1 mes.
- Se pueden añadir al cultivo inhibidores de la Transcriptasa Inversa y de la proteasa. En el caso de estos últimos se necesitan al menos de 72h de infección para ver su efecto.

Protocolo basado en las siguientes referencias:

Bleiber et al; 2001, JVI.

Simon et al, 2003, JVI.

# ANEXO II

Otras publicaciones

## Listado de otras publicaciones

J.Martinez-Picado, K. Morales-Lopetegi, T Wein, JG Prado, S Frost, CJ Petropolous, B Clotet, L Ruiz.
Selection of drug-resistant HIV-1 mutants in response to repeated structured treatment interruptions.
**AIDS 2002 Apr 12;16(6):895-9**.

Lidia Ruiz, Esteban Ribera, Anna Bonjoch, Joan Romeu, Javier Martínez-Picado, Roger Paredes, Marjorie Díaz, Silvia Marfil, Eugenia Negredo, Julia GPrado, Cristina Tural, Guillem Sirera and Bonaventura Clotet.
Role of structured treatment interruption before a 5-drug salvage antiretroviral regimen: the Retrogene Study.
**J Infect Dis. 2003 Oct 1;188(7):977-85**.

Prado JG, Shintani A, Bofill M, Clotet B, Ruiz L, Martinez-Picado J.
Lack of longitudinal intrapatient correlation between p24 antigenemia and levels of human immunodeficiency virus (HIV) type 1 RNA in patients with chronic hiv infection during structured treatment interruptions.
**J Clin Microbiol. 2004 Apr;42(4):1620-5**.

Draenert R, Le Gall S, Pfafferott KJ, Leslie AJ, Chetty P, Brander C, Holmes EC, Chang SC, Feeney ME, Addo MM, Ruiz L, Ramduth D, Jeena P, Altfeld M, Thomas S, Tang Y, Verrill CL, Dixon C, Prado JG, Kiepiela P, Martinez-Picado J, Walker BD, Goulder PJ.
Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection.
**J Exp Med. 2004 Apr 5;199(7):905-15**.

Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, Feeney M, Tang Y, Holmes EC, Allen T, Prado JG, Altfeld M, Brander C, Dixon C, Ramduth D, Jeena P, Thomas SA, St John A, Roach TA, Kupfer B, Luzzi G, Edwards A, Taylor G, Lyall H, Tudor-Williams G, Novelli V, Martinez-Picado J, Kiepiela P, Walker BD, Goulder PJ
HIV evolution: CTL escape mutation and reversion after transmission.
**Nat Med. 2004 Mar;10(3):282-9**.

# Selection of drug-resistant HIV-1 mutants in response to repeated structured treatment interruptions

**Javier Martinez-Picado[a], Kristina Morales-Lopetegi[a], Terri Wrin[b], Julia G. Prado[a], Simon D.W. Frost[c], Christos J. Petropoulos[b], Bonaventura Clotet[a] and Lidia Ruiz[a]**

**Background** A new HIV-1 treatment strategy based on repeated structured treatment interruptions (STI) is currently being evaluated in clinical trials to determine whether immune cell-mediated control of viral replication can be stimulated by intermittent periods of viral replication. The potential for selection of drug-resistant quasi-species remains a major concern of such a treatment strategy.

**Methods** Plasma and peripheral blood lymphocyte (PBL) samples from 12 patients who had three consecutive STIs were studied. Genotypic analysis was based on population and clonal sequencing. Drug susceptibility and their corresponding replication capacities were evaluated by a single-cycle growth assay.

**Results** Consistent with a loss of phenotypic susceptibility to lamivudine, the M184V mutation was detected by genotypic analysis (direct and clonal sequencing) in plasma samples collected from two patients at the end of the second or third STI. Longitudinal analysis of patient samples revealed a step-wise increase in the M184V mutation in each patient virus population over successive STIs, despite the lower replicative capacity associated with this mutation in the absence of antiviral agents.

**Conclusion** Drug-resistant virus can rise to high frequencies in chronically HIV-1 infected individuals during consecutive STIs. Evolution of resistance is likely to be more important in patients with prior suboptimal therapies, particularly when few mutations are required for resistance. Maximum care should be taken in designing STI protocols that minimize development of drug-resistant mutations that may lead to treatment failure. Thus, drug-resistance testing may be useful before restarting treatment during STI studies.



*AIDS* 2002, **16**:895–899

**Keywords: HIV-1, antiviral drug resistance, structured treatment interruption**

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MAJOR ARTICLE

# Role of Structured Treatment Interruption before a 5-Drug Salvage Antiretroviral Regimen: The Retrogene Study

**Lidia Ruiz,[1] Esteban Ribera,[2] Anna Bonjoch,[1] Joan Romeu,[1] Javier Martinez-Picado,[1] Roger Paredes,[1] Marjorie Díaz,[2] Silvia Marfil,[1] Eugenia Negredo,[1] Julia García-Prado,[1] Cristina Tural,[1] Guillem Sirera,[1] and Bonaventura Clotet[1]**

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**We evaluated the efficacy of a 5-drug salvage regimen, preceded by a 12-week, structured treatment interruption (STI), in 46 multidrug-treated, human immunodeficiency virus type 1–infected patients with detectable viremia. Patients were randomly assigned to receive a 5-drug salvage regimen immediately (noninterruption [NI] group; $n = 24$ patients) or after 12 weeks of STI (interruption [I] group; $n = 22$ patients). At week 48, 45% of patients in the I group and 46% of patients in the NI group had virus loads <50 HIV-1 RNA copies/mL ($P = .619$). No differences in CD4 cell counts were seen between groups at week 48 ($P = .734$). A complete reversion to wild-type genotype was detected in 35% of patients in the I group, but this phenomenon did not affect the virological response. The only overall baseline factor associated with ensuing virus suppression was a lower number of nucleoside reverse-transcriptase inhibitor–resistant mutations (relative risk, 0.66; 95% confidence interval, 0.47–0.93; $P = .021$). A prior STI seems to confer no additional benefit to subsequent virological or immunological outcomes of a salvage regimen.**

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# Lack of Longitudinal Intrapatient Correlation between p24 Antigenemia and Levels of Human Immunodeficiency Virus (HIV) Type 1 RNA in Patients with Chronic HIV Infection during Structured Treatment Interruptions

Julia G. Prado,[1] Ayumi Shintani,[2] Margarita Bofill,[1] Bonaventura Clotet,[1] Lidia Ruiz,[1] and Javier Martinez-Picado[1]*

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**Structured treatment interruptions (STIs) have been proposed as a potential treatment strategy during human immunodeficiency virus type 1 (HIV-1) antiretroviral therapy. This still-experimental intervention requires a close monitoring of patients' plasma viremia and $CD4^+$-T-cell counts during the treatment interruption phase. By using signal amplification of a heat-dissociated p24 antigen (p24Ag) assay, we compared p24Ag levels with levels of HIV RNA in plasma. One hundred seventy-four plasma samples were obtained from 51 chronically HIV-infected patients: 117 from patients who underwent STIs and 57 from patients who did not. Partial immune complex dissociation and clearance of those complexes by the erythrocytes were also investigated. A significant association between the two assays was observed ($\beta = 0.23$, 95% confidence interval = 0.18, 0.28; $P < 0.0001$), but the association was smaller in the subset of samples from patients undergoing STIs. Moreover, discordant results and lack of longitudinal intrapatient correlation between levels of p24Ag and HIV-1 RNA were higher in this group. Incomplete immune complex dissociation and binding of those complexes to erythrocytes could be contributing factors involved in the diminished detection of p24Ag. Therefore, signal amplification of a heat-dissociated p24Ag had a positive association with current HIV RNA assays in a population-based analysis. However, it might not be sensitive enough to monitor longitudinal intrapatient viremia during STIs in patients with high $CD4^+$-T-cell counts potentially due to the production of high-affinity anti-p24 antibodies and clearance of immune complexes by erythrocytes.**

# Immune Selection for Altered Antigen Processing Leads to Cytotoxic T Lymphocyte Escape in Chronic HIV-1 Infection

Rika Draenert,[1] Sylvie Le Gall,[1] Katja J. Pfafferott,[2] Alasdair J. Leslie,[2] Polan Chetty,[3] Christian Brander,[1] Edward C. Holmes,[7] Shih-Chung Chang,[5] Margaret E. Feeney,[1] Marylyn M. Addo,[1] Lidia Ruiz,[6] Danni Ramduth,[3] Prakash Jeena,[4] Marcus Altfeld,[1] Stephanie Thomas,[3] Yanhua Tang,[1] Cori L. Verrill,[1] Catherine Dixon,[2] Julia G. Prado,[6] Photini Kiepiela,[3] Javier Martinez-Picado,[6] Bruce D. Walker,[1] and Philip J.R. Goulder[1,2]

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## Abstract

Mutations within cytotoxic T lymphocyte (CTL) epitopes impair T cell recognition, but escape mutations arising in flanking regions that alter antigen processing have not been defined in natural human infections. In human histocompatibility leukocyte antigen (HLA)-B57$^+$ HIV-infected persons, immune selection pressure leads to a mutation from alanine to proline at Gag residue 146 immediately preceding the $NH_2$ terminus of a dominant HLA-B57–restricted epitope, ISPRTLNAW. Although N-extended wild-type or mutant peptides remained well-recognized, mutant virus–infected CD4 T cells failed to be recognized by the same CTL clones. The A146P mutation prevented $NH_2$-terminal trimming of the optimal epitope by the endoplasmic reticulum aminopeptidase I. These results demonstrate that allele-associated sequence variation within the flanking region of CTL epitopes can alter antigen processing. Identifying such mutations is of major relevance in the construction of vaccine sequences.

Key words: CD8 T cell responses • viral evolution • immune evasion • antigen presentation

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# HIV evolution: CTL escape mutation and reversion after transmission

A J Leslie[1, 12], K J Pfafferott[1, 12], P Chetty[2], R Draenert[3], M M Addo[3], M Feeney[3], Y Tang[3], E C Holmes[4], T Allen[3], J G Prado[5], M Altfeld[3], C Brander[3], C Dixon[1], D Ramduth[2], P Jeena[2], S A Thomas[2], A St John[6], T A Roach[6], B Kupfer[7], G Luzzi[8], A Edwards[9], G Taylor[10], H Lyall[10], G Tudor-Williams[10], V Novelli[11], J Martinez-Picado[5], P Kiepiela[2], B D Walker[3] & P J R Goulder[1, 3]

**Within-patient HIV evolution reflects the strong selection pressure driving viral escape from cytotoxic T-lymphocyte (CTL) recognition. Whether this intrapatient accumulation of escape mutations translates into HIV evolution at the population level has not been evaluated. We studied over 300 patients drawn from the B- and C-clade epidemics, focusing on human leukocyte antigen (*HLA*) alleles *HLA-B57* and *HLA-B5801*, which are associated with long-term HIV control and are therefore likely to exert strong selection pressure on the virus. The CTL response dominating acute infection in *HLA-B57/5801*-positive subjects drove positive selection of an escape mutation that reverted to wild-type after transmission to *HLA-B57/5801*-negative individuals. A second escape mutation within the epitope, by contrast, was maintained after transmission. These data show that the process of accumulation of escape mutations within HIV is not inevitable. Complex epitope- and residue-specific selection forces, including CTL-mediated positive selection pressure and virus-mediated purifying selection, operate in tandem to shape HIV evolution at the population level**

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Una página en blanco, tengo toda una página en blanco, para mi enterita y nadie me la puede quitar. Puedo poner todo lo que quiera y vaya si caben palabras!!! no corregiré las faltas de ortografía y no pondré puntos, que cada uno lo lea como quiera. Algunos dicen que esta es la pagína más morbosa de toda la tesis, y por eso, al igual que ocurre con el periódico, hay gente que comienza leer por el final a lo que algunos autores contestan con un texto breve e indiferente, yo no, tengo que dar las gracias y son de verdad:

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