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Analyzing complex resistance patterns of PIs with bio-informatics resistance determination (BIRD): a novel approach employing synthetic viral genes carrying clinically relevant patterns of PI-resistance mutations

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ABSTRACT

Background: The increasing incidence of HIV-1 protease inhibitor (PI) cross-resistance necessitates the better understanding of the molecular basis of resistance development to currently marketed as well as investigational PIs. The aim of this study is to investigate the differential impact of multi-drug resistance (MDR) associated mutations involved in resistance to such drugs.

Methods: A novel bio-informatics resistance determination (BIRD) approach was established. An analysis of our in-house database of matching phenotypic and genotypic profiles of HIV-1 clinical isolates was performed, which yielded a defined set of multi-PI resistant viruses (sources) and a corresponding set of genetically closely related PI susceptible viruses (targets). For each source we constructed a set of synthetic protease genes carrying all combinations of resistance mutations that differ between source and target, representing multiple genetic pathways. Those synthetic gene products were used for recombinant protein and/or virus production to build a multidimensional dataset, which helps to delineate the genetic pathways involved in HIV-1 resistance to PIs.

Results: In a proof-of-concept study, we selected two source viruses to construct a set comprising 40 synthetic protease gene variants carrying the primary PI mutations L33F, M46I, I50V, I84V, and L90M in a background of resistant associated mutations L10I, K20I, M36I, I47V, I54L, A71V, and V77I, in different combinations. Removal of the mutations L33F, A71V from their sources had a significant impact on viral growth resulting in an impaired viability. Subsequent phenotypic characterization (21/40 viruses) showed that L33F, I47V, and I54L in this PI-resistance genetic background played a crucial role in reduced susceptibility to marketed PIs (e.g. amprenavir and lopinavir). In addition we observed that the binding affinity (surface plasmon resonance) between such PIs and MDR proteases was often reduced, mainly as a result of increased dissociation rates.

Conclusion: The BIRD approach elaborates on genetic profiles observed in the clinic and allows the assessment of the influence of all combinations of mutations in complex, PI-resistant genetic background. Future work includes measurement of binding kinetics between mutant proteases and PIs. This novel approach will guide our further understanding of the molecular basis for resistance development to current and investigational PIs.

INTRODUCTION

- HIV-1 protease is a homodimeric aspartic protease with a central, symmetric substrate-binding cavity. This enzyme is responsible for cleaving the gag and gag-pol polyproteins into the structural and functional proteins necessary for the proper assembly and maturation of infectious virions.
- The introduction of PIs into the HIV treatment armamentarium in 1995 led to a remarkable decline in HIV-1-related morbidity and mortality.[1] PIs are now a cornerstone of highly active antiretroviral therapy (HAART).
- All currently approved PIs utilize the same chemical framework to inhibit the HIV-1 protease and therefore extensive cross-resistance can occur between PIs. In order to design drugs with higher genetic barriers to resistance, we need to fully understand the molecular basis of how resistance develops.
- In this study, we describe the novel bio-informatics resistance determination (BIRD) approach used to investigate the isolated impact of mutations associated with complex PI multi-drug resistance patterns. Here, we use the development of the novel PI TMC114 as an example of the effective application of BIRD.

METHODS

- An analysis of an in-house database of matching phenotypic and genotypic profiles of HIV-1 clinical isolates yielded a set of multi-PI-resistant viruses (sources) and a corresponding set of genetically closely related PI-susceptible viruses (targets).
- For each source, a set of synthetic protease genes carrying all combinations of resistance mutations that differ between source and target was designed. By keeping the background mutations from the source, a set of synthetic genes per source-target pair was obtained. In a proof-of-concept study, two such sets were selected for synthesis, which yielded a total of 40 synthetic genes.
- Before synthesizing the genes, their sequences were processed to create sets of overlapping oligonucleotides of specified lengths, and were padded with BamHI and EcoRI restriction sites designed for the direct cloning into the pBluescript® (Stratagene, US) II KS (+) vector.
- This step was followed by chemical synthesis of each oligonucleotide with GeneWriter™ (Egea, US) technology. Oligonucleotides were analyzed and purified by 12% PAGE. Using GeneAssembler™ (Egea, US) technology, combinatorial subset libraries were assembled and cloned into the pBluescript® (Stratagene, US) II KS (+) vector.
- *E. coli* electro-competent Dh5a cells were transformed with all sets. Clones were then picked and sequenced on an ABI 3730xl DNA sequencer. The inserts were re-amplified with nested polymerase chain reaction. The resulting amplicons were homologously recombined into a proviral clone, and the resulting recombinant viruses were harvested, titrated, and used to test in-vitro susceptibility to PIs (as described in Hertogs et al.[2] and modified for Enhanced Green Fluorescent Protein [EGFP]-based fluorescent read-out).
- To determine virus titers and viral growth, MT4 cells equipped with Long Terminal Repeat-Enhanced Green Fluorescent Protein (LTR-EGFP) at 500,000 cells/mL, were inoculated with different dilutions of the recombinant viruses. The plates were incubated at 37°C and 5% CO_2 and fluorescence of the wells was read at different timepoints post infection.

RESULTS

Identification of sources and targets

- An in-house database of clinical HIV-1 isolates was analyzed for viruses that showed a reduced susceptibility for TMC114 (fold change [FC] ≥40; Figure 1). These viruses were selected to be sources in the analysis, and their genotype was determined. For each source, a corresponding clinical isolate with a closely related genotype but a divergent phenotype was identified.
- While the susceptibility of the target isolates for TMC114 was much higher (FC <4), their gag-pro-RT sequence was very similar to that of the source, differing by a few resistance mutations. For proof of concept, two source viruses were selected and two corresponding susceptible target viruses were identified.

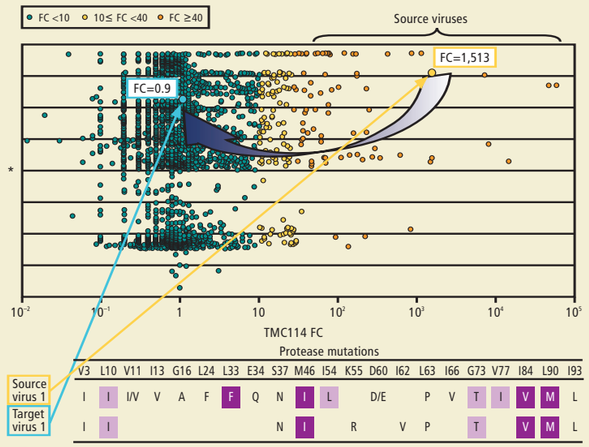

Figure 1. Selection of source 1 (reduced sensitivity for TMC114: FC ≥40) and target 1 (high TMC114 susceptibility FC <10) clinical isolates with closely related genotypes.

Design of intermediate genotypes

- A set of synthetic protease genes carrying all combinations of resistance mutations that differ between source and target was constructed (Tables 1 and 2). This exercise resulted in the design of 40 synthetic genes, which were used to generate recombinant viruses.
- In this study, 50% of the recombinant viruses had a high enough titer to generate a measurable EGFP signal after 3 days of incubation (Tables 1 and 2). Signals of slower growing but viable viruses were not measured.

Influence of point mutations on phenotype

- In the intermediate genotypes between source virus 1 and target virus 1 the titers for viruses (–L33F) and (–L33F, –V77I) were too low for viral growth. Consistently, removal of the L33F mutation in this background caused a reduced or immeasurable viral growth (Table 1).
- The contribution of the protease mutations to TMC114 sensitivity in these different intermediate genotypes, in a background of primary PI-resistance mutations L33F, M46I, I84V and L90M, is shown in Figure 2
  - the contribution of the V77I mutation seemed to be negligible
  - the contribution of I54L to the susceptibility of the source virus was about 6-fold
  - the impact of the L33F mutation on susceptibility was ~6-fold in the absence of I54L.
- Figure 3 shows how another set of resistance mutations, between source virus 2 and target virus 2, influences PI susceptibility (amprenavir, lopinavir, TMC114)
  - removing the A71V mutation in this background reduced viral growth resulting in immeasurable growth (Table 2). Hence, the impact of the A71V mutation on in-vitro susceptibility to amprenavir, lopinavir and TMC114 could not be assessed
  - amprenavir and lopinavir sensitivity of the intermediate genotypes showed that almost all viruses show FC values ≥10 (Figure 3a)
  - for amprenavir and lopinavir the contribution to reduced susceptibility (defined by increase in FC) was largest for I47V, followed by L10I and to a lesser extent K20I, while the contribution of M36I seemed to be negligible
  - TMC114 sensitivity of the intermediate genotypes showed that the contribution of the I47V mutation (in a background of primary PI resistance mutations M46I and I50V) to the reduced susceptibility of the source virus was about 10-fold, while the contribution of K20I and M36I seemed to be negligible (Figure 3b)
  - the influence of the L10I mutation was less clear, but seemed to cause a ~2-fold reduction in TMC114 susceptibility.
- These results are in line with findings in the clinic.[3]

Table 1. Design of the intermediate genotypes between source virus 1 and target virus 1.

ID	Protease mutations: V3	L10	V11	I13	G16	L24	L33	E34	S37	M46	I54	K55	D60	I62	L63	I66	G73	V77	I84	L90	I93	Removed mutations from source virus	Viral growth
Source virus 1	I	I	I/V	V	A	F	F	Q	N	I	L		D/E		P	V	T	I	V	M	L		+
SGMP001648	I	I		I	V	A	F	F	Q	N	I	L		E		P	V	T	I	V	M	L		+
SGMP001649	I	I		I	V	A	F	F	Q	N	I			E		P	V	T	I	V	M	L	–I54L	+
SGMP001650	I	I		I	V	A	F		Q	N	I	L		E		P	V	T	I	V	M	L	–L33F	–
SGMP001652	I	I		I	V	A	F	F	Q	N	I	L		E		P	V	T		V	M	L	–V77I	+
SGMP001651	I	I		I	V	A	F		Q	N	I			E		P	V	T	I	V	M	L	–L33F –I54L	±
SGMP001653	I	I		I	V	A	F	F	Q	N	I			E		P	V	T		V	M	L	–I54L, –V77I	+
SGMP001654	I	I		I	V	A	F		Q	N	I	L		E		P	V	T		V	M	L	–L33F, –V77I	–
SGMP001655	I	I		I	V	A	F		Q	N	I			E		P	V	T		V	M	L	–L33F, –I54L, –V77I	±
Target virus 1	I	I								N	I		R		V	P		T		V	M	L		+

Primary PI mutations
Resistance-associated PI mutations
Removed mutation

Table 2. Design of the intermediate genotypes between source virus 2 and target virus 2.

ID	Protease mutations: V3	L10	I13	Q18	K20	M36	S37	M46	I47	I50	Q61	L63	H69	A71	I72	L76	I93	Removed mutations from source virus	Viral growth
Source virus 2	I	I	V	H	I	I	N	I	V	V	H	P		V	V	V			+
SGMP001156	I	I	V	H	I	I	N	I	V	V	H	P		V	V	V			+
SGMP001160	I		V	H	I	I	N	I	V	V	H	P		V	V	V		–L10I	+
SGMP001164	I	I	V	H		I	N	I	V	V	H	P		V	V	V		–K20I	+
SGMP001172	I	I	V	H	I	I	N	I	V	V	H	P			V	V		–A71V	–
SGMP001157	I	I	V	H	I	I	N	I		V	H	P		V	V	V		–I47V	+
SGMP001158	I	I	V	H	I		N	I	V	V	H	P		V	V	V		–M36I	+
SGMP001168	I		V	H		I	N	I	V	V	H	P		V	V	V		–L10I, –K20I	±
SGMP001176	I		V	H	I	I	N	I	V	V	H	P			V	V		–L10I, –A71V	–
SGMP001161	I		V	H	I	I	N	I		V	H	P		V	V	V		–I47V, –L10I	++
SGMP001162	I		V	H	I		N	I	V	V	H	P		V	V	V		–M36I, –L10I	+
SGMP001180	I	I	V	H		I	N	I	V	V	H	P			V	V		–K20I, –A71V	–
SGMP001165	I	I	V	H		I	N	I		V	H	P		V	V	V		–I47V, –K20I	++
SGMP001166	I	I	V	H			N	I	V	V	H	P		V	V	V		–M36I, –K20I	++
SGMP001173	I	I	V	H	I	I	N	I		V	H	P			V	V		–I47V, –A71V	+
SGMP001174	I	I	V	H	I		N	I	V	V	H	P			V	V		–M36I, –A71V	–
SGMP001159	I	I	V	H	I		N	I		V	H	P		V	V	V		–I47V, –M36I	+
SGMP001184	I		V	H		I	N	I	V	V	H	P			V	V		–L10I, –K20I, –A71V	–
SGMP001169	I		V	H		I	N	I		V	H	P		V	V	V		–I47V, –L10I, –K20I	±
SGMP001170	I		V	H			N	I	V	V	H	P		V	V	V		–M36I, –L10I, –K20I	+
SGMP001177	I		V	H	I	I	N	I		V	H	P			V	V		–I47V, –L10I, –A71V	±
SGMP001178	I		V	H	I		N	I	V	V	H	P			V	V		–M36I, –L10I, –A71V	–
SGMP001163	I		V	H	I		N	I		V	H	P		V	V	V		–I47V, –M36I, –L10I	+
SGMP001181	I	I	V	H		I	N	I		V	H	P			V	V		–I47V, –K20I, –A71V	±
SGMP001182	I	I	V	H			N	I	V	V	H	P			V	V		–M36I, –K20I, –A71V	–
SGMP001167	I	I	V	H			N	I		V	H	P		V	V	V		–I47V, –M36I, –K20I	++
SGMP001175	I	I	V	H	I		N	I		V	H	P			V	V		–I47V, –M36I, –A71V	–
SGMP001185	I		V	H		I	N	I		V	H	P			V	V		–I47V, –L10I, –K20I, –A71V	–
SGMP001186	I		V	H			N	I	V	V	H	P			V	V		–M36I, –L10I, –K20I, –A71V	–
SGMP001171	I		V	H			N	I		V	H	P		V	V	V		–I47V, –M36I, –L10I, –K20I	+
SGMP001179	I		V	H	I		N	I		V	H	P			V	V		–I47V, –M36I, –L10I, –A71V	–
SGMP001183	I	I	V	H			N	I		V	H	P			V	V		–I47V, –M36I, –K20I, –A71V	±
SGMP001187	I		V	H			N	I		V	H	P			V	V		–I47V, –M36I, –L10I, –K20I, –A71V	–
Target virus 2	I						D	I		V		P	H/Y			L			+

Primary PI mutations
Resistance-associated PI mutations
Removed mutation

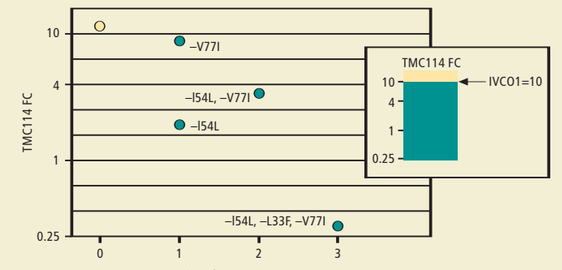

Figure 2. Influence of removing mutations from source virus 1 on TMC114 susceptibility (IVCO = in-vitro cut-off).

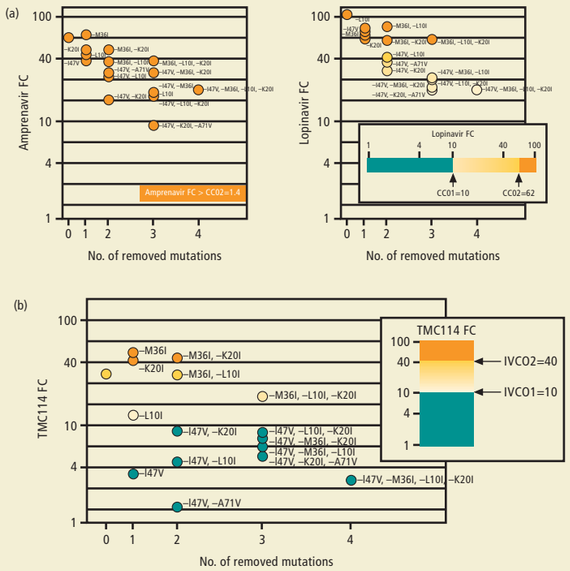

Figure 3. Influence of removing mutations from source virus 2 on susceptibility of a) amprenavir and lopinavir (CCO: clinical cut-off), and b) TMC114 (IVCO = in-vitro cut-off).

CONCLUSIONS

- The BIRD approach elaborates on genetic profiles observed in clinical HIV isolates and allows the assessment of the influence of all combinations of mutations in complex, PI-resistant genetic background. Future work includes measurement of binding kinetics between mutant proteases and PIs.
- This novel approach will guide our further understanding of the molecular basis for resistance development to current (e.g. amprenavir, lopinavir) and investigational PIs (e.g. TMC114).

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