J Antimicrob Chemother 2021; **76**: 130–134
doi:10.1093/jac/dkaa401 Advance Access publication 24 September 2020

Journal of Antimicrobial Chemotherapy

In vitro cross-resistance to doravirine in a panel of HIV-1 clones harbouring multiple NNRTI resistance mutations

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Received 19 May 2020; accepted 28 August 2020

Objectives: Doravirine is a recently licensed HIV-1 NNRTI with improved efficacy, pharmacokinetics and safety profile compared with efavirenz and limited cross-resistance with rilpivirine and etravirine. In this *in vitro* study, cross-resistance to doravirine was analysed in a representative panel of NNRTI-resistant clones.

Methods: *In vitro* phenotypic susceptibility to doravirine was assessed in 10 clinically derived infectious clones with intermediate- to high-level resistance to rilpivirine, etravirine, efavirenz and nevirapine, and in NL4-3 site-directed mutants harbouring K103N, Y181C, M230L or K103N/Y181C NNRTI mutations.

Results: Although none of the infectious clones harboured any of the major doravirine resistance-associated mutations (RAMs) included in the IAS-USA reference list, doravirine fold change (FC) values were comparable to or higher than those calculated for other NNRTIs, particularly etravirine and rilpivirine. As expected, single NNRTI mutations K103N and Y181C did not impair doravirine susceptibility (FC 1.4 and 1.8, respectively), while reduced activity was observed with the single M230L or double K103N/Y181C mutations (FC 7.6 and 4.9, respectively). Median FC values increased significantly with increasing numbers of NNRTI RAMs ($P = 0.005$) and were >10 in 4/4 and 1/4 clones harbouring four and three NNRTI RAMs, respectively. FC values correlated well with predicted susceptibility as inferred by Stanford HIV Drug Resistance Database (HIVdb) and ANRS algorithms (both $P < 0.001$).

Conclusions: Substantial cross-resistance to doravirine was detected in NNRTI-resistant viruses harbouring complex mutational patterns, even in the absence of major IAS-USA doravirine RAMs. Therefore, based on the simple IAS-USA reference list, doravirine resistance may be underestimated in viruses harbouring multiple NNRTI mutations.

Introduction

Doravirine (formerly MK-1439) is a novel once-daily NNRTI approved by the US FDA for the treatment of HIV-1 in therapy-naive patients or as a switch option in virologically suppressed patients with no history of treatment failure and no known substitutions associated with resistance to doravirine.[1] The approval label of the EMA provided slightly different therapeutic indications, recommending the use of doravirine for the treatment of adults infected with HIV-1 without past or present evidence of resistance to the NNRTI class.[2] In clinical studies, doravirine showed non-inferior efficacy and improved pharmacokinetics and/or safety profile, both as a switch option in virologically suppressed patients[3] and as part of a first-line regimen, when compared with efavirenz and darunavir.[4,5] Moreover, doravirine was effective in halting viral replication even in the presence of transmitted NNRTI mutations, such as K103N and G190A, in a small group of treatment-naive patients.[6] Based on *in vitro* and *in vivo* data, the last update of the IAS-USA HIV-1 drug resistance mutations list indicates V106A/M and Y188L as major doravirine mutations and V106I/T, Y188C/H, G190E, P225H, F227C/L/R, M230L and L234I as minor mutations.[7] Indeed, *in vitro* studies demonstrated that doravirine retained full activity against most of the single resistance-associated mutations (RAMs) selected by older NNRTIs, except for V106A, Y188L and M230L, as well as against some combinations of multiple NNRTI RAMs.[8,9] A recent study conducted on a large panel of clinical isolates collected from treatment-naive patients revealed that 92.5% of samples were susceptible to doravirine, as indicated by a fold change (FC) value lower than the biological cut-off of 3-fold.[10] *In vitro* resistance selection experiments revealed the emergence of V106A/M/I, V108I, F227C/I/L and L234I, with minimal HIV-1 subtype-related differences,[11] resulting in a limited cross-resistance with rilpivirine and possibly with etravirine.[12] Although three recent large surveys showed a low prevalence of doravirine RAMs in NNRTI-exposed individuals,[13–15] the impact of various combinations of NNRTI RAMs and the possible cross-resistance with other

NNRTIs have been poorly investigated. Importantly, one clinical trial (NCT04233216) has recently started to recruit heavily treatment-experienced patients with multidrug-resistant viruses harbouring NNRTI and NRTI RAMs to evaluate the efficacy of the doravirine/islatravir combination plus optimized background therapy. In this study, we aimed to evaluate the activity of doravirine in a reference panel of NNRTI-resistant infectious clones and in site-directed mutants including relevant NNRTI mutations.

Materials and methods

Cell lines

Lenti-X 293 T cells (Takara Bio, Kusatsu, Japan) and TZM-bl cells were cultured in high-glucose DMEM with L-glutamine, supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The MT-2 cell line was cultured in RPMI supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. TZM-bl and MT-2 cell lines were obtained from the Centre for AIDS Reagent of the National Institute for Biological Standards and Control. All cell culture media and relevant reagents were obtained from EuroClone (Italy).

NNRTI-resistant infectious clones

A reference panel of HIV-1 infectious clones harbouring combinations of major NNRTI RAMs was obtained from the NIH AIDS Reagent Program. These clinically derived recombinant viruses were characterized by intermediate- to high-level resistance to rilpivirine, etravirine, efavirenz and nevirapine as determined by the Phenosense Assay.[16] Reverse transcriptase sequences of NNRTI-resistant clones were submitted to GenBank under accession numbers JQ814884–JQ814893. In addition, we introduced the individual NNRTI mutations K103N, Y181C, M230L and the combination of K103N/Y181C into the HIV-1 NL4-3 backbone through the QuikChange® Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The NL4-3 plasmid was obtained from the NIH AIDS Reagent Program. NL4-3 and all clones with NNRTI mutations were transfected into Lenti-X 293 T cells, propagated in MT-2 cells and titrated in TZM-bl cells as previously described.[17]

Phenotypic determination of susceptibility to doravirine

In vitro susceptibility to doravirine was determined in duplicate through a TZM-bl cell-based assay previously shown to correlate well with the reference phenotypic Phenosense Assay in the measurement of susceptibility to HIV-1 protease, reverse transcriptase and integrase inhibitors.[17] Briefly, 10 000 TZM-bl cells/well were infected with the WT NL4-3 strain or NNRTI-resistant viruses at a multiplicity of infection of 0.03 in the presence of 5-fold dilutions of doravirine (MedChemExpress, Monmouth Junction, NJ, USA) ranging from 10 μM to 0.00512 nM. After 48 h, cells were treated with the Glo-Lysis buffer (Promega, Madison, WI, USA) and the Bright-Glo Luciferase Assay (Promega), then relative luminescence units were measured through the GloMax Discover instrument (Promega) and elaborated with GraphPad software to calculate IC_{50} values. FC values were calculated with respect to the IC_{50} value obtained with the NL4-3 WT strain.

Results and discussion

According to the IAS-USA drug resistance mutations list,[7] none of the clones harboured any major doravirine RAMs (namely V106A/M and Y188L), while two clones included the minor doravirine RAM M230L. As described in Table 1, 8 out of the 10 NNRTI-resistant clones and the mutant K103N/Y181C NL4-3 clone have an FC value higher than the biological cut-off. The highest FC values (>100) were found in samples 12225 and 12237, harbouring mutations E138G/H221Y/F227L/M230L and V106I/Y181C/G190A/H221Y, respectively, while the other sample harbouring M230L together with L100I and V179D (ID 12243) showed an FC value of 6.2. Other clones with Y181C and additional NNRTI RAMs (ID 12231, 12235 and 12239) had FC values of 14.2–31.8, suggesting a considerably reduced susceptibility to doravirine, while the absence of Y181C and doravirine RAMs (clones 12227, 12229, 12233 and 12241) resulted in FC values from 0.4 to 3.1, suggesting no or minimal impact on doravirine susceptibility. The single K103N or Y181C mutations within the NL4-3 backbone did not reduce doravirine susceptibility (FC values 1.4 and 1.8, respectively), while the double mutant K103N/Y181C showed a reduced susceptibility (FC 4.9). These data were comparable to those already described for viruses harbouring single K103N or Y181C and the K103N/Y181C mutations, showing mean FC values of 1.5, 2.5 and 4.3, respectively.[8] Interestingly, clone 12231 harbouring mutations K103N/V179F/Y181C showed an FC value of 22.1, suggesting that the non-polymorphic V179F substitution by itself or in combination with other uncharacterized amino acid variations can further decrease doravirine susceptibility. Similarly, the site-directed NL4-3/M230L mutant showed an FC value of 7.6, comparable to those observed in clone 12243 but significantly lower than in clone 12225, harbouring the additional doravirine RAM F227L together with NNRTI RAMs E138G and H221Y. Overall, doravirine FC values were comparable to or lower than those calculated with other licensed NNRTIs in clones 12227, 12229, 12233, 12241 and 12243, while in the remaining five clones doravirine FC values were higher than those of other NNRTIs, particularly etravirine and rilpivirine. Irrespective of the type of NNRTI RAMs, a higher number of NNRTI RAMs correlated significantly with increasing median FC values (linear test for trend, $P = 0.005$) (Figure 1a). Indeed, all of the eight viruses with ≥3 NNRTI mutations showed FC values >3, including clones 12231, 12235, 12237 and 12239 with no canonical doravirine mutations. By comparing FC values and predicted susceptibility to doravirine, both the HIV Drug Resistance Database (HIVdb; version 8.9-1) and the ANRS algorithm (version 30) could estimate doravirine activity with good accuracy, indicating that these two algorithms can be reliably used to evaluate doravirine susceptibility for possible use in patients harbouring NNRTI RAMs (Figure 1b and c).

The 10 clinically derived NNRTI-resistant clones were originally conceived to include combinations of NNRTI mutations commonly observed among sequences stored in the Stanford HIVdb and causing intermediate- to high-level resistance to nevirapine, efavirenz, rilpivirine and etravirine.[16] This study completes the NNRTI susceptibility profile for this reference panel of clones, which is publicly available and ideal to inform further NNRTI development. Partly contrary to expectations, we found that doravirine activity was overall similar to that of the second-generation NNRTIs etravirine and rilpivirine, indicating that doravirine may only partially overcome NNRTI resistance. While previous *in vitro* testing had been mostly carried out on viruses harbouring one or two NNRTI mutations,[8,9] the key result of this study is that multiple mutations selected by older NNRTIs can confer substantial cross-resistance to doravirine even in the absence of major IAS-USA doravirine RAMs. It must also be noted that clones derived from clinical isolates accommodate *in vivo* selected minor or compensatory changes which cannot be recapitulated in site-directed mutants,

Table 1. Mean IC_{50} ± SD, fold change values for doravirine and other licensed NNRTIs against infectious clones harbouring NNRTI resistance-associated mutations (RAMs) and levels of doravirine activity predicted by the Stanford HIVdb and ANRS prediction algorithms

Virus ID	NNRTI RAMs	NRTI RAMs	Other mutations	Mean doravirine IC_{50} ± SD (nM)	Fold change (FC)					Predicted doravirine activity[d]	
					Doravirine[b]	Nevirapine[c]	Efavirenz[c]	Etravirine[c]	Rilpivirine[c]	Stanford HIVdb	ANRS
12225[a]	E138G, H221Y, F227L, M230L	M41L, L210W, T215Y	K122E, D177E, I178L, R211K, V245M, I293V	>100	>100	>200	15	21	18	R	R
12227[a]	K101P, K103N	M41L, T215Y	A98S, K102Q, D123E, K166R, D177E, D192N, R211K, V245K, K277R, R284K, T286A, E297K	0.4 ± 0.1	0.4	>200	>200	5.8	92	S	S
12229[a]	L100I, K103N, H221Y	M41L, L74V, M184V, T215Y	R83K, D177E, T200A, R211Q, K281R, R284K, I293V, E297A	4.8 ± 0.3	4.0	>200	>200	6.8	6.3	I	R
12231[a]	K103N, V179F, Y181C	M41L, T215F	K49R, K82R, A98S, D177E, G196E, Q207K, R211A, L228R, A272P, I293V, K311R	26.5 ± 14.4	22.1	>200	90	8.8	2.3	I	R
12233[a]	K101E, Y181V	K70R, M184V, T215F	T27S, E28K, K32E, V60I, T69N, R83K, V179I, T200A, Q207D, L228R, V245E, T286A, E297K	3.3 ± 0.5	2.8	>200	2.1	27	24	I	I
12235[a]	A98G, K101E, Y181C, G190A	M41L, E44D, D67N, T69D, L74I, L210W, T215Y	T39E, V118I, K122E, I135T, G196E, E203K, R211K, A272S, V276T, K277R, Q278E, L283I, I293V, E297K	38.2 ± 5.8	31.8	>200	>200	15	22	R	R
12237[a]	V106I, Y181C, G190A, H221Y	none	V35I, S68G, A98S, D121E, K122E, I135V, R211K, F214L, V245E, D250E, A272P, E297K	>100	>100	>200	26	6	3.5	R	R
12239[a]	A98G, K101E, E138K, Y181C	M41L, T215D	K122E, I135T, S162Y, T200A, L210F, P243T, V245E, D250E, A272P, I274V, Q278H, K281R, T286A, A288S, K311R	17.0 ± 4.0	14.2	>200	3.6	10	9.2	I	R
12241[a]	K101E, E138G, G190S	none	V35M, R211K, V245E, S251I	3.8 ± 1.4	3.1	>200	>200	3.2	2.6	I	R
12243[a]	L100I, V179D, M230L	M41L, D67G, L74I, M184V, T215Y	V35I, K103R, K122E, I202V, R211K, T240K	7.5 ± 0.6	6.2	>200	>200	95	13	R	R
NL4-3/103N	K103N			2.0 ± 1.2	1.4	NA	NA	NA	NA	S	S
NL4-3/181C	Y181C			2.6 ± 1.1	1.8	NA	NA	NA	NA	S	S
NL4-3/ 103N/181C	K103N, Y181C			6.9 ± 2.8	4.9	NA	NA	NA	NA	I	R
NL4-3/230L	M230L			10.6 ± 4.7	7.6	NA	NA	NA	NA	R	R

NA, not available.
[a]NIH AIDS Reagent Program catalogue number.
[b]Doravirine FC values calculated according to the NL4-3 strain IC_{50} value of 1.4±0.7 nM.
[c]FC values determined through the Phenosense Assay.[16]
[d]The HIVdb five-level grading was collapsed to three levels as indicated by the HIValg release notes at https://hivdb.stanford.edu/page/release-notes/. S, susceptible; I, intermediate resistance; R, high-level resistance.

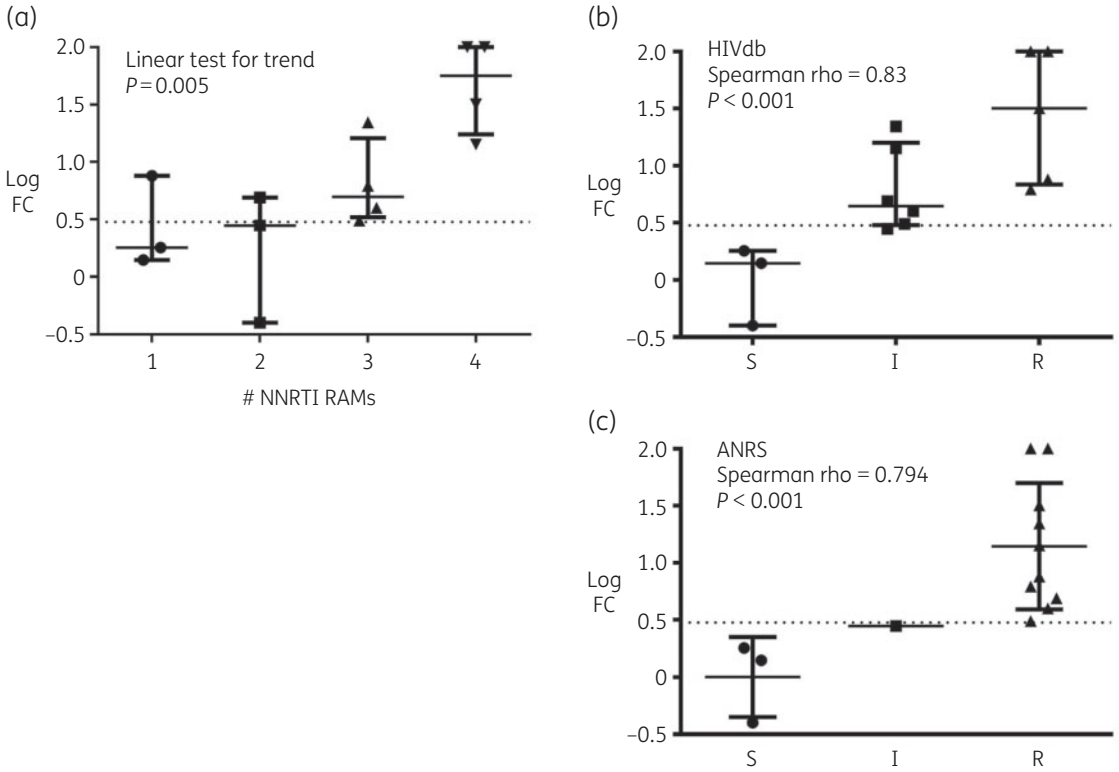

Figure 1. Distribution of doravirine fold change (FC) values measured with clones harbouring NNRTI resistance-associated mutations (RAMs), as grouped by (a) number of NNRTI RAMs (linear test for trend, $P = 0.005$); (b) predicted doravirine susceptibility by the Stanford HIVdb algorithm; (c) predicted doravirine susceptibility by the ANRS algorithm. S, susceptible; I, intermediate resistance; R, high-level resistance. The dotted line indicates the biological FC cut-off (3-fold) recently established for doravirine.[10]

yet may play a relevant role in modulating resistance. For example, patient-derived viruses harbouring G190S or L100I + K103N without any canonical doravirine mutation showed FC values in the range of 1.5–11 and 2.7–19, respectively.[8]

A relevant consequence of these findings is that prediction of resistance simply based on the presence of IAS-USA doravirine RAMs may overestimate doravirine activity. Genotypic interpretation systems such as the Stanford HIVdb or ANRS should be preferred in the context of multiple NNRTI mutations. Indeed, a recent study showed that the Stanford HIVdb detected more transmitted resistance to doravirine with respect to the IAS-USA list.[15] Importantly, clinical use of doravirine as part of salvage therapy in heavily treatment-experienced patients still needs to be informed by expanded *in vitro* genotype–phenotype correlation analysis, coupled with *in vivo* data allowing the establishment of clinical FC cut-offs.

Acknowledgements

This study was presented at the 17th European AIDS Conference, 6–9 November 2019, Basel, Switzerland, abstract PS5/6.

Funding

The authors acknowledge the contribution of the CARE Consortium funded by the European Union's Horizon 2020 programme and the Ministry of Science and Higher Education of the Russian Federation. In addition, the study was partially supported by ViiV Healthcare for the project 'HIV multidrug resistance pathways in EuResist Integrated DataBase'.

Transparency declarations

M.Z. reports consultancy fees for ViiV Healthcare, Gilead Sciences and Janssen-Cilag, and grants for his institution from ViiV Healthcare and Gilead outside the submitted work. R.W.S. received research funding from Janssen Pharmaceuticals and Vela Diagnostics outside the submitted work. All other authors have none to declare.

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