Prevalence and *in vitro* characteristics of Reverse Transcriptase (RT) N348I Mutation in Non-B subtypes in the Absence of Viral Load Monitoring, in the DART Study (NORA substudy)

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Poster #643

INTRODUCTION

The N348I mutation in the connection domain of HIV-1 reverse transcriptase contributes to reduced susceptibility to nucleoside and non-nucleoside reverse transcriptase inhibitors.

The prevalence of N348I in subtype B viruses is around 9% in those failing NNRTI containing regimens. We studied this mutation in non subtype B viruses in patients enrolled in the NORA study- a randomised double-blinded trial of CBV/NVP vs CBV/ABC conducted in Uganda, in the absence of viral load monitoring, a setting typical of ART rollout programmes.

The impact of N348I mutation alone or in combination with other mutations in RT on drug susceptibility and viral fitness was studied in a range of non subtype B viruses using a novel single cycle retroviral vector system that includes entire *gag* and *pol* sequences.

METHODS

Study Population to measure prevalence of N348I:

NORA is a 600 patient nested substudy of the DART trial in Uganda, in which drug naïve patients were randomized to combivir plus Abacavir (ABC) or Nevirapine (NVP). Plasma virus was extracted from patients with VL>1000 copies/ml at wk96 and at baseline and the *pol* gene sequenced. Ie: 47 patients on CBV/ABC and 24 patients on CBV/NVP.

Mutagenesis and Phenotypic drug susceptibility assay:

Site-directed mutagenesis was performed to introduce the N348I mutation alone or in combination with K65R or M184V mutations in RT for HIV-1 subtypes A, B, C and D.

Subtype specific *gag-pol* sequences were amplified from molecular clones: P8.9NSX (B) MJ4 (C), 94UG114.1.6 (D), and subtype A virus was derived from a drug naïve patient infected with HIV-1. Both wild type (WT) and mutant sequences were cloned into a replicative defective, envelope deleted subtype B vector (P8.9 NSX).

Virus stocks were generated by co-transfection of HEK 293T cells with relevant mutant or wild type subtype specific gag-pol expression vector, a plasmid encoding a luciferase reporter gene flanked by HIV-1 LTRs and including a packaging signal, and a plasmid encoding VSV-G envelope to infect target cells. Virus produced is then capable of a single infectious event.

Virus was harvested 48hrs post-transfection and used to infect target HEK 293T cells in the presence of drug. The IC_{50} was determined by "sigmoidal dose-response, variable slope" nonlinear regression analysis of the percentage inhibition of virus versus $\log_{10}$ drug concentration using GraphPad Prism 5 software.

Viral fitness assay:

Replication capacity (RC) was determined by titrating virus 48hrs post-infection and measuring luciferase expression from infected target cells in the absence of drug. RC values for mutant virus were calculated from the amount of Relative Light Units (RLU) per ng p24 input and were expressed as a percentage of wild type.

RESULTS

1. N348I mutation was detectable in 10/71 patients failing first line HAART at 96wks on CBV plus ABC or NVP and was associated with NRTI and NNRTI resistant mutations.

N348I mutation was detected in similar prevalence in patients failing on CBV/ABC (6/47; 13%) or CBV/NVP (4/24; 17%) first line HAART at 96 weeks, and was associated with M184V mutation in all instances or with one or more TAMs (Table 1) . The N348I mutation was not detectable as a polymorphism in associated baseline samples and was not confined to a particular subtype.

Subtype	Ist line HAART	NRTI mutations	NNRTI mutations
D	CBV + NVP	M184V T215Y	K101E G190A
C	CBV + NVP	M184V T215F	Y181V
D	CBV + NVP	K70R M184V	V108I Y181C
A	CBV + NVP	M41L M184V L210W T215F	Y181C
D	CBV + ABC	D67N K70R M184V T215Y K219E	NONE
D	CBV + ABC	T69I M184V L210W T215F	NONE
C	CBV + ABC	M184V	NONE
D	CBV + ABC	D67N K70R M184V K219Q	NONE
D	CBV + ABC	M184V T215Y	NONE
A	CBV + ABC	D67N K70R M184V T215F K219E	NONE

Table 1. Associations with N348I (N=71). None of the patients had substituted drugs/switched to second-line before 96 weeks.

2. N348I mutation confers reduced NRTI and NNRTI drug susceptibility in B and non- B HIV-1 isolates .

Phenotypic drug susceptibility testing was performed on wild type and N348I mutant viruses (subtypes A , B, C and D) to ascertain the impact of N348I on drug resistance. The N348I mutation alone confers reduced susceptibility to all NNRTIs tested.

RT Drug Inhibitor	Subtype A	Subtype B	Subtype C	Subtype D
D4T	0.86 ± 0.08	1.07 ± 0.04	0.91 ± 0.30	1.17 ± 0.06
AZT	2.8 ± 2.02	2.66 ± 0.44	2.56 ± 0.70	1.40 ± 0.25
3TC	1.26 ± 0.11	1.36 ± 0.13	1.12 ± 0.48	1.18 ± 0.07
DDI	0.99 ± 0.12	1.10 ± 0.09	0.73 ± 0.61	1.26 ± 0.17
ABC	1.28 ± 0.65	1.01 ± 0.16	0.78 ± 0.18	1.13 ± 0.24
FTC	1.28 ± 0.14	1.34 ± 0.22	1.63 ± 1.09	1.36 ± 0.23
TDF	1.07 ± 0.16	1.22 ± 0.15	1.30 ± 0.37	1.13 ± 0.16
NVP	5.10 ± 2.38	4.40 ± 0.87	5.88 ± 1.94	2.87 ± 0.30
EFV	3.13 ± 0.38	2.46 ± 0.33	2.23 ± 0.24	2.42 ± 0.09
ETV	2.6 ± 0.51	2.38 ± 0.56	1.90 ± 0.51	3.08 ± 0.39

Table 2. Effect of N348I on NRTI and NNRTI resistance. Data are mean fold changes in IC_{50} of mutant compared to wild type ± Std dev obtained from 3 independent experiments.

3. N348I mutation reduces replicative capacity (RC) in HEK 293T cells lines for B and non- B HIV-1 subtypes compared to wild type virus.

	Subtype A	Subtype B	Subtype C	Subtype D
HEK 293T	47.27%	56.35%	74.26%	89.00%

Table 3. Effect of N348I on viral fitness. RC values for mutant virus are expressed as a % of wild type virus.

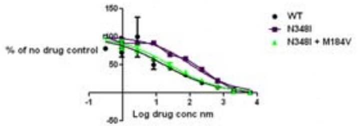
4. Impact of N348I plus M184V on (A) AZT drug resistance (B) RC across B and non- B HIV-1 subtypes.

Since all participants in our NORA substudy possessed N348I mutation along with the M184V mutation, we performed phenotypic assays on non-B viruses containing these two mutations to study their effect on drug resistance and fitness.

The presence of N348I alone confers AZT resistance, but having both N348I and M184V on the same viral genome, reverses this effect. Figure 1A illustrates the resistance pattern for subtype C virus, and occurs across the other subtypes (data not shown), but having a more profound effect in subtype C virus (4.40 fold decrease in IC_{50}) compared with 2.24, 2.69, and 1.88 fold decrease in IC_{50} for AZT in subtypes A, B and D respectively.

The addition of the M184V mutation does not impact on viral fitness that is observed for N348I alone . This pattern is common for all subtypes (Figure 1B).

Figure 1A : Effect of N348I alone or plus M184V on AZT resistance for subtype C virus.



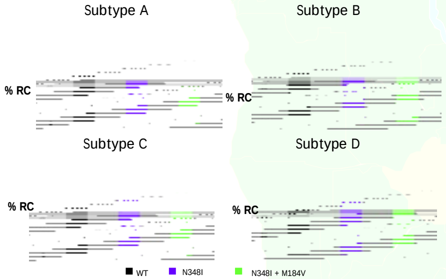


Figure 1B. Effect of N348I alone or plus M184V on viral fitness. RC values for mutant virus were calculated from the amount of Relative Light Units (RLU) per ng p24 input and were expressed as a percentage of wild type.

Statistical analyses from a UK Resistance Database/CHIC on 2194 drug treatment experienced individuals revealed that the N348I mutation was rarely associated with the K65R mutation (number of patients with K65R= 125/2194 (5.7%), number of patients with K65R and N348I=1 /2194 (p=0.001) .

We performed drug susceptibility and fitness assays on mutant virus containing N348I in the presence and absence of K65R mutation to address if there is any bidirectional antagonism between the two mutations when they exist on the same viral genome.

5. Impact of N348I mutation plus K65R on drug resistance- K65R reverses drug resistance to NNRTIs conferred by N348I alone.

Drug susceptibility assays reveal that the fold increase in IC_{50} we observe for NVP, EFV, ETV and AZT in N348I mutant virus is reversed with the addition of the K65R mutation, and this trend occurs across all the HIV-1 subtypes.

However, not surprisingly, resistance to ABC, DDI, 3TC, FTC and TDF is increased in the N348I K65R double mutant.

Drug	Subtype A		Subtype B		Subtype C		Subtype D	
	N348I	N348I+K65R	N348I	N348I+K65R	N348I	N348I+K65R	N348I	N348I+K65R
D4T	1.99	1.80	1.38	2.40	1.67	2.70	0.95	1.89
AZT	3.13	1.44	2.84	1.36	6.34	4.91	1.84	1.64
3TC	0.92	10.32	1.06	13.16	0.91	21.42	0.98	15.40
DDI	0.82	2.15	1.36	3.64	1.02	3.04	0.98	2.61
ABC	1.03	1.94	1.24	2.46	1.34	2.61	1.11	2.32
FTC	1.09	7.34	1.24	9.64	1.19	11.58	1.18	8.19
TDF	1.45	2.42	1.24	2.06	1.27	4.66	0.92	2.77
NVP	4.97	3.05	3.93	2.97	4.25	3.00	3.79	1.51
EFV	3.97	1.44	2.87	1.62	3.26	1.69	2.96	1.01
ETV	3.15	1.36	2.34	1.72	2.04	1.00	2.82	0.86

Table 4. Effect of N348I alone or combined with K65R on NRTI and NNRTI resistance. Data are mean fold changes in IC_{50} of mutant compared to wild type from one experiment.

6. N348I plus K65R reduces viral fitness in non-B HIV-1 subtypes.

Viral fitness is reduced in N348I mutant virus compared with wild type virus across all subtypes, and N348I mutant virus is made less fit when combined with the K65R mutation for subtypes A, C and D, with a minimal effect on subtype B virus.

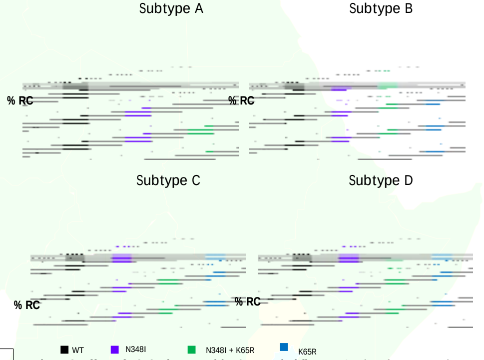


Figure 2. Effect of N348I alone or with K65R on viral fitness RC values for mutant virus were calculated from the amount of Relative Light Units (RLU) per ng p24 input and were expressed as a percentage of wild type.

* The level of p24 was too low to quantify for K65R mutation alone in subtype A virus.

CONCLUSIONS

- •We observe a similar prevalence for selection of the N348I mutation with NRTI or NNRTI containing 1st line drug regimens.
- •N348I mutation was co-selected with M184V and TAMs in most patients.
- •N348I mutation increases resistance to NNRTIs across B and non-B HIV-1 isolates. The Fold change to Etravirine we observe for N348I mutant virus may reach the level that is required to compromise its activity.
- •However, we have only studied the impact of N348I mutation *in vitro* in isolation, or with K65R or M184V, rather than with all the mutations found in our NORA patients.
- •N348I knocks down replicative capacity for all subtypes. We do observe some subtype differences in N348I impact on viral fitness and resistance to certain drugs.
- •The impact of N348I mutation on response to second line agents remains unclear and is likely to be determined by other co-existing mutations.

We thank all the patients and staff from all the centres participating in the DART trial.

MRC Programme on AIDS/Uganda Virus Research Institute, Entebbe, Uganda: H Grosskurth, P Munderi, G Kabuye, D Nsibambi, R Kasirye, E Zalwango, M Nakazibwe, B Kikaire, G Nassuna, R Massa, K Fadhiru, M Namyalo, A Zalwango, L Generous, P Khauka, N Rutikarayo, W Nakahima, A Mugisha, J Todd, J Levin, A Ruberantwari, P Hughes, M Aber. **Joint Clinical Research Centre, Kampala, Uganda:** P Mugyenyi, C Kityo, D Tumukunde, F Ssali, D Atwine, G Mulindwa, R Byaruhanga, T Bakeimyaga-Grace, H Katabira, E Nimwesiga, G Barungi, S Atwiine, F Ahimbisibwe, S Tugume, T Otim, J Takubwa, M Mulindwa, S Murungi, J Tukamushaba, D Muebesa, H Kyomugisha, J Kagina, P Katundu, O Labeja. **University of Zimbabwe, Harare, Zimbabwe:** A Latif, J Hakim, V Robertson, A Reid, E Chidziva, A Jamu, S Makota, R Bulaya-Tembo, G Musoro, N Ngorima, F Taziwa, L Chakonza, H Chirairo, S Chitsungo, F Mapinge, A Mawora, C Muvirimi, G Tinago, J Chimanzi, J Machingura, C Maweni, S Mutsai, R Warara, M Matongo, S Mudzingwa, M Jangano, K Moyo, L Vere, M Phiri, Bafana T. **Academic Alliance, Mulago Hospital, Uganda:** E Katabira, J Oyugi, A Ronald, A Kambungu, R Nalumenya, F Sematala, R Nairubi, E Bulume, M Teopista, C Twijukye, Lubwana E. **The AIDS Support Organisation (TASO), Uganda:** A Coutinho, B Etukoit. **Imperial College , London, UK:** C Gilks, K Boocock, C Puddephatt, D Winogron. **MRC Clinical Trials Unit, London, UK:** J Darbyshire, DM Gibb, A Burke, D Bray, A Babiker, AS Walker, H Wilkes, M Rauchenberger, L Peto, K Taylor, M Spyer, A Ferrier, B Naidoo. **Independent DART Trial Monitors:** R Nanfuka, C Mufuka-Kapuya. **Trial Steering Committee:** I Weller (Chair), A Babiker (Trial Statistician), S Bahendeka, M Bassett, A Chogo Wapakhabulo, J Darbyshire, B Gazzard, C Gilks, H Grosskurth, J Hakim, A Latif, C Mapuchere, O Mugurungi, P Mugyenyi; Observers: C Burke, S Jones, C Newland, S Rahim, J Rooney, M Smith, W Snowden, J-M Steens. **Data and Safety Monitoring Committee:** A Breckenridge (Chair), A McLaren (Chair-deceased), C Hill, J Matenga, A Pozniak, D Serwadda. **Endpoint Review Committee:** T Peto (Chair), A Palfreeman, M Borok, E Katabira. **Funding:** DART is funded by the **UK Medical Research Council**, the **UK Department for International Development (DFID)**, and the **Rockefeller Foundation**. **GlaxoSmithKline, Gilead** and **Boehringer-Ingelheim** donated first-line drugs for DART, and **Abbott** provided LPV/r (Kaletra/Aluvia) as part of the second-line regimen for DART. **Virology Group:** P Awio, A Burke, M Chirara, D Dunn, D Gibb, C Gilks, R Goodall, H Grosskurth, J Hakim, P Kaleebu, P Katundu, C Kityo, F Lyagoba, A McCormick, P Mugyenyi, P Munderi, N Ndembi, D Pillay, A Reid, V Robertson, S Tugume, D Yirrell.