Understanding patterns of HIV multi-drug resistance through models of temporal and spatial drug heterogeneity

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1 Abstract

Triple-drug therapies have transformed HIV from a fatal condition to a chronic one. These therapies should prevent HIV drug resistance evolution, because one or more drugs suppress any partially-resistant viruses. In practice, such therapies drastically reduced, but did not eliminate, resistance evolution. In this article, we reanalyze published data from an evolutionary perspective and demonstrate several intriguing patterns about HIV resistance evolution - resistance evolves (1) at an approximately constant rate each year after an initial period, (2) via one mutation at a time and (3) in a predictable order. We describe how these observations might emerge under two models of HIV drugs varying in space or time. Despite decades of work in this area, much opportunity remains to create models with realistic parameters for three drugs, and to match model outcomes to resistance rates and genetic patterns from patients on triple-drug therapy. Further, lessons from HIV may inform other systems.

2 Introduction

Better anti-retroviral therapies have drastically improved the lifespan of individuals living with HIV (Marcus et al., 2020) by preventing the evolution of drug resistance (Zazzi et al., 2018). When the first HIV drugs were introduced, drug resistance evolved in nearly all patients in the first 6 months of treatment (Larder et al., 1989), and sometimes within weeks (Schuurman et al., 1995). This drug resistance, often encoded by single mutations conferring strong resistance (Larder and Kemp, 1989; Günthard et al., 2019), led to rebounding viral populations and treatment failure.

Triple-drug therapies, introduced in 1995, were expected to prevent the evolution of drug resistance and subsequent treatment failure (Hammer et al., 1997). The reasoning behind this is as follows - each of three drugs individually brings the expected number of viruses produced by a single infected cell (the basic reproductive number, R_0) below one, causing the viral population to shrink. Even if a virus gets a mutation conferring resistance to one drug, the two other drugs keep the R_0 of that virus below one, and so viruses carrying this mutation cannot increase in number (Nowak and May, 2000). This limits the spread of single drug resistance mutations and requires that multiple mutations be present for a virus to replicate successfully in the presence of three drugs and cause population growth. Despite HIV's high mutation rate, the probability of multiple mutations emerging simultaneously in a single round of replication is exceedingly small (Perelson and Nelson, 1999). While triple-drug combination therapies saved many lives (Hogg et al., 1999; Walensky et al., 2006), HIV has continued to evolve drug resistance into the modern day (Lee et al., 2014; Rocheleau et al., 2018).

How HIV can evolve resistance to triple-drug combination therapies represents an enduring mystery. Advances in our understanding of drug resistance evolution on triple-drug therapy are driven mostly by two

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types of studies. First, analysis of clinical patient data has revealed which drug resistance mutations occur (Günthard et al., 2019), how often resistance evolution happens on different drug combinations (Lee et al., 2014; Rocheleau et al., 2018), and how patient behavior and metabolism correlate with the probability of virologic failure (Harrigan et al., 2005). Second, mathematical models have explored how heterogeneity of drug levels in time (Rong et al., 2007; Braithwaite et al., 2006; Rosenbloom et al., 2012; Cadosch et al., 2012; Bershteyn and Eckhoff, 2013) and space (Kepler and Perelson, 1998; Moreno-Gamez et al., 2015; Sanche et al., 2017) can allow the evolution of resistance, and make certain treatments more likely to fail. While some of these models are quite sophisticated, few consider combinations of three drugs and stochastic emergence of resistance, and there is no common framework to test the effects of spatial and temporal heterogeneity on triple-drug combinations of antiretrovirals.

While no model will ever be sufficiently detailed to capture all the nuances of virus-host-drug interactions in a human body, we argue that a significant gap remains in understanding how multi-drug resistance emerges, as revealed by various facets of clinical trial and cohort study data. Matching models specifically to mutational patterns in clinical data can help us better understand whether these models can explain multi-drug resistance evolution in the real world.

In this perspective, we describe several features of drug resistance evolution in HIV, as observed from clinical data, that we believe have not received the attention they deserve. We then describe two types of models that are good candidates for explaining the observations. The important feature of these models is that they include heterogeneity of selection pressures - either in time or in space. We close by suggesting a path forward in expanding promising mathematical models of HIV to understand three-drug combinations and testing the models' ability to match clinical data across multiple dimensions. We also discuss how we think the lessons from HIV can be applied in other systems.

2.1 Two motivating examples

We first describe two studies that report particularly detailed data on how drug resistance emerges across many individuals with HIV, Kempf et al. (2004) and Hoffmann et al. (2009). Like most modern day therapies, these studies examine drug combinations pairing two nucleoside reverse transcriptase inhibitors (a so-called NRTI therapy backbone) with a drug from a different class - either a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI). These studies paint a detailed picture of resistance evolution, in that they record both when resistance emergence and which relevant mutations are present.

Kempf study: In a clinical trial (M98-863) conducted internationally, 327 participants were randomized to receive treatment with two NRTIs, 3TC (lamivudine) and D4T (stavudine), and a protease inhibitor, NFV (nelfinanvir) (Kempf et al., 2004). Patient outcomes from this clinical trial are plotted in Figure 1A. We highlight three observations. First, many viral populations evolved drug resistance. Within the first 2 years of therapy, drug resistance was detected in 79 of the 327 patients (24%). Kaplan-Meier plots in the original paper show that roughly half of these cases occurred in the first year of treatment and half in the second year – indicating that standing genetic variation at the start of treatment was not exclusively responsible for treatment failure. Second, drug-resistant viruses were often not resistant to all three drugs in a treatment. Out of these 79 patients, approximately half carried resistance to one drug, half carried resistance to two drugs, and 8 patients had resistance to three drugs. Third, drug resistance mutations occurred in a predictable order. All 35 patients with resistance to one drug had resistance to 3TC and 35 of 36 patients with resistance to two drugs had resistance to 3TC and the PI (NFV). These data therefore strongly suggest that on 3TC+D4T+NFV treatment, drugs fail in a consistent order: 3TC resistance arises first, then NFV resistance, and finally D4T resistance (see Figure 1A).

Hoffman study: A study in South Africa followed patients treated with a common NNRTI-based regimen containing two NRTI drugs, AZT (zidovudine) and 3TC, and one NNRTI, EFV (efavirenz) (Hoffmann et al., 2009). Outcomes for these patients are plotted in Figure 1B. Most of the patients experiencing virologic failure who were genotyped (48/68) had drug resistance mutations. However, similar to the Kempf trial, many of these resistant viral populations had only partially resistant genotypes. Of these genotyped patients, 21 had resistance to just one drug, 20 had resistance to two drugs, and 3 had resistance to all three drugs. Again, the partially resistant populations tended to have predictable compositions: 19 of the singly resistant populations were resistant to EFV (the NNRTI) and just 2 had resistance to 3TC. All patients resistant to two drugs had resistance to EFV and 3TC. These data therefore strongly suggest that on 3TC+AZT+EFV

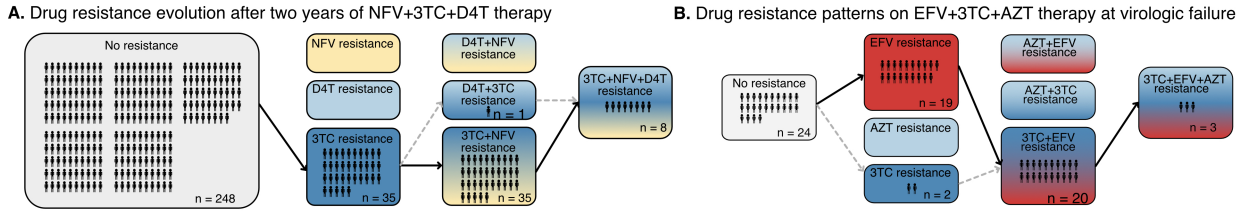

Figure 1: **A. Kempf study:** Resistance to the drug 3TC emerges first among patients with HIV taking the triple-drug cocktail NFV + 3TC + D4T (n=327) in Kempf et al. (2004). NFV is a protease inhibitor, and D4T and 3TC are nucleoside reverse transcriptase inhibitors. **B. Hoffman study:** Resistance to the drug EFV emerges first among patients with HIV taking the triple-drug cocktail EFV + 3TC + AZT (n=68) in Hoffmann et al. (2009). EFV is a non-nucleoside reverse transcriptase inhibitor, and AZT and 3TC are nucleoside reverse transcriptase inhibitors. Abbreviations: 3TC: lamivudine, AZT: zidovudine, D4T: stavudine, EFV: efavirenz, NFV: nelfinanvir. Solid lines (followed from left to right) represent the most frequent progression of drug resistance, whereas dashed lines represent observed minority paths to resistance.

treatment, drugs again fail in a consistent order, although a different order from the PI-based treatment. Here, EFV failed first (Figure 1B), and 3TC only second, whereas in Kempf et al. (2004), 3TC consistently failed first (Figure 1A).

It is well-known that 3TC resistance can evolve extremely quickly when 3TC is used as a monotherapy (Feder et al., 2021). 3TC resistance also evolves first when it is co-administered with AZT (Picard et al., 2001; Larder et al., 1995) and as we showed in the previous paragraph, it evolves first when co-administered with D4T and the PI NFV. It is therefore somewhat unexpected that when co-administered with an NNRTI, it appears to not be selected unless NNRTI resistance has already appeared.

Several consistent observations emerge from these two studies: 1) significant resistance evolves despite triple-drug treatments. 2) Viruses do not need to be fully resistant to all drugs in a combination in order to spread. 3) Resistance mutations emerge in a partially predictable order. As we discuss below, all three of these patterns are observed much more broadly in larger datasets.

3 Three key observations of HIV drug resistance evolution during triple-drug therapy

In this section, we look at bigger, yet less detailed, datasets to determine if the observations from the case studies (resistance evolution is ongoing, sequential, and with a predictable order) hold more generally.

3.1 Rates of drug resistance evolution are approximately constant after extended time on therapy.

Despite triple-drug therapies becoming available in high-income countries in the 1990s, drug resistance evolution remained common. Resistance emerged not only in patients who had started their treatment before 1995 on mono- or double-drug therapies, but also in patients who started on a triple-drug regimen in the 1990s or later. Specifically, resistance evolution occurred both in NNRTI-based and PI-based treatments. In addition, several studies (Rocheleau et al., 2018; on HIV Drug Resistance et al., 2005), following cohorts of patients over time found that after the first year or two of treatment, drug resistance evolution occurs at a *surprisingly constant rate* (Figure 2A). Even patients on treatment for 5 or more years remain at risk for drug resistance evolution highlighting the importance of ongoing within-patient processes in treatment failure and suggesting that resistance patterns are not driven by a subset of patients who fail therapy quickly. Figure 2A is redrawn from an extensive study out of British Columbia in which all HIV-infected people in the province were automatically entered in a provincial registry and received treatment from a central agency free of charge (Montaner et al., 2010). The figure plots data from 2692 patients and shows ongoing evolution of resistance for four drug classes over 10 years for patients enrolling between 1996 and 2005 (Rocheleau

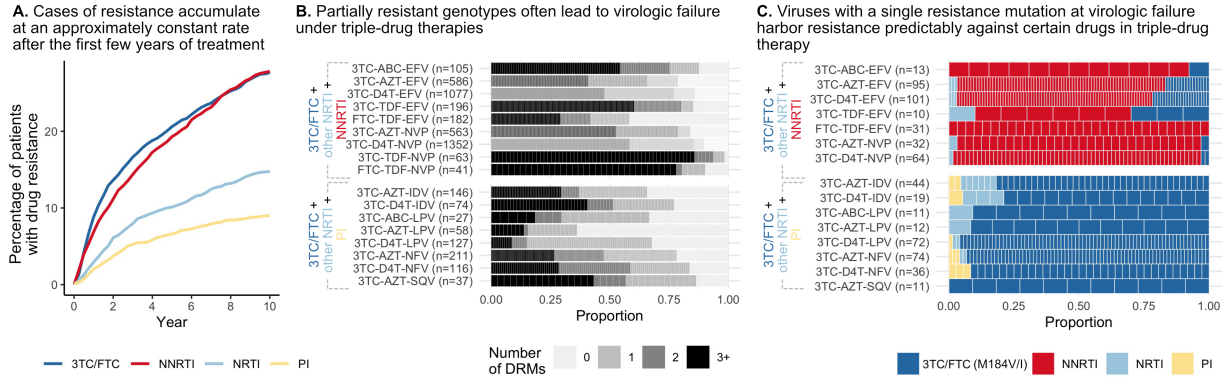

Figure 2: **A.** After the first two years of treatment, the probability of acquiring a drug resistance mutation is approximately constant, leading to an increase in drug resistance over time in a cohort study of Canadians with HIV sampled in between 1996 and 2004 (figure replotted from Rocheleau et al. (2018)). The percentage of patients detected with resistance is plotted separately for resistance to 3TC or FTC (dark blue), NNRTIs (red), NRTIs other than 3TC or FTC (light blue) or PIs (yellow). **B.** Across patients treated with three-drug combination therapies based on two NRTIs paired with an NNRTI (top) or PI (bottom), many sequences are detected with 0, 1, 2 or 3+ drug resistance mutations in a large cohort of patients (data source: Feder et al. (2016)). **C.** In patients treated with three-drug combination therapies based on two NRTIs paired with an NNRTI (top) who have exactly one resistance mutation, that mutation most often confers resistance to the NNRTI of the treatment (red), although sometimes resistance to 3TC (dark blue) or the other NRTI (light blue) develops. Among patients treated with a PI paired with 3TC and a second NRTI who have a single drug resistance mutation, that mutation most often confers resistance to 3TC (dark blue), although sometimes it confers resistance to the other NRTI (light blue) or the PI (yellow) (Data source: Feder et al. (2016)). Each square in both **B** and **C** represents a single patient, and the sample sizes are given next to the treatment names. Abbreviations: abacavir, ABC; indinavir, IDV; lamivudine, 3TC; lopinavir, LPV; nelfinanvir, NFV; stavudine, D4T; saquinavir, SQV; zidovudine, AZT.

et al., 2018). Importantly, the fraction of patients with resistant virus increases essentially linearly after year two, suggesting that the risk of drug resistance evolution on triple-drug therapy remains even in patients who have been on successful treatment for years.

3.2 Partially-resistant genotypes lead to virologic failure

Patients on HIV treatment in high income countries are tested regularly for viral load. If the viral load is higher than a certain threshold ("viremia"), a viral sample will be sequenced and assessed for resistance mutations (DHHS, 2006). Observing sequences with only one or two resistance mutations at this stage suggests that partial resistance can allow growth above the detectable viral load threshold. This is surprising under how we believe triple-drug therapies should work, since a singly-resistant virus should be suppressed by two other drugs and not expand in the population.

In earlier papers, we have mentioned the observation that drug resistance evolves in a step-wise fashion (Pennings et al., 2014; Pennings, 2013), but did not systematically query this effect across a broad range of triple-drug therapies. To understand if this effect is widespread across treatments, we analyzed a large set of HIV sequences (6717 patients) from the Stanford HIV Drug Resistance Database. The presented sequences stem from samples at detection of viremia on triple-drug therapy. In these sequences, we identified large-effect drug resistance mutations and quantified the number of mutations per sequence (Figure 2B, see methods). While some patients failed without any detectable drug resistance (likely due to non-adherence), viral populations in most patients harbored one, two, three or more resistance mutations. That many sequences harbored only one or two drug resistance mutations suggests that partially resistant virus can replicate in the body and lead to a detectable viral load, even when treated with tree drugs. This is consistent with results from Figure 1 showing that resistance mutations accumulate in a seemingly step-wise fashion. Note, viruses with three or more resistance mutations may still have accumulated resistance

mutations one at a time but these intermediate steps may not have been captured by sampling.

3.3 Drug resistance mutations accumulate in a predictable order

Possibly the most surprising observation from our two cases studies is that drug resistance mutations emerge in a particular order. Ordering of mutations in HIV has been noted before for different mutations that confer drug resistance to the same drug (Beerenwinkel et al., 2005b,a; Posada-Céspedes et al., 2020). That resistance to one class of drugs tends to precede resistance to another class of drugs has not received much attention in the literature. To understand which resistance mutations emerge first on triple-drug therapy, we examined only viral populations which harbored a single resistance mutation in Figure 2B. For each of those singly-resistant populations, we determined the drug to which the mutation conferred resistance (Figure 2C). In patients on PI-based therapies, the singly-resistant viral populations almost all carry a 3TC mutation, suggesting that resistance to 3TC overwhelmingly occurs before resistance to either the other NRTI or the PI. This ordering of mutations cannot be explained by different mutational target sizes or mutation rates. For example, the product of the mutation rate and target size for PI resistance is twice that for 3TC resistance ($1.7 \cdot 10^{-4}$ for PIs such as NFV and LPV, vs $8 \cdot 10^{-5}$ for 3TC, see Methods), yet 3TC resistance occurs first in about 90% of the cases (Figure 2C). In addition, the 3TC mutation M184V incurs a substantial fitness cost (Paredes et al., 2009; Jain et al., 2011), which should make it unlikely to be present as standing genetic variation (Simen et al., 2009). In patients treated with NNRTI based therapies, the first drug resistance mutation confers resistance most often to NNRTIs (Figure 2C), which means that NNRTI resistance occurs first.

Collectively, we have presented three observations that can help guide the construction of models: (1) drug resistance evolution was quite common on triple-drug therapies in the 1990s and 2000s, and even patients that have been on treatment for several years are still at risk for resistance evolution; (2) resistance evolves in a sequential manner (first one mutation, then a second, then a third, rather than three mutations at the same time), leading to the observation of partially-resistant viruses; (3) mutations emerge in a predictable order that cannot be explained by mutational target size. We next describe two potential explanations for drug resistance evolution on triple-drug therapies and explore how they might produce the patterns described above.

4 Two models of resistance evolution due to drug level heterogeneity

Two types of models have been used to explain how drug resistance emerges on combination therapies: models with temporal heterogeneity in drug concentrations (Rong et al., 2007; Braithwaite et al., 2006; Rosenbloom et al., 2012; Cadosch et al., 2012; Bershteyn and Eckhoff, 2013) and models with spatial heterogeneity in drug concentrations (Kepler and Perelson, 1998; Moreno-Gamez et al., 2015; Sanche et al., 2017). Both models rely on drugs falling below their intended concentrations at certain points in time or space. The literature on temporal heterogeneity is more extensive and includes cohort studies on the adherence-resistance relationship in HIV (Bangsberg et al., 2004; Harrigan et al., 2005). While nobody doubts that spatial and temporal heterogeneity exist, it is not clear if either or both can explain the observed evolutionary patterns.

Before describing in more detail the spatial and temporal heterogeneity models, we first explain why heterogeneity is crucial - namely, HIV drugs work too well at their administered concentrations for ongoing replication. Even when administered as monotherapy, drugs cause sensitive viral populations to shrink (Eron et al., 1995; Reiss et al., 1988). As an example, we plot data from a patient treated with AZT monotherapy (Figure 3A). Initially, the treated viral population shrinks. Only after 16 weeks, the viral population expands again to detectable levels. Measuring the ability of the drug-resistant and sensitive virus to replicate at different drug concentrations (i.e., the Hill dose-response curve, Figure 3B, Chou (1976); Zhang et al. (2004)) helps explain the dynamics.

At certain intermediate drug concentrations, the resistant mutant can grow ($R_0 > 1$) and the sensitive strain cannot ($R_0 < 1$) (Rosenbloom et al. (2012), Figure 3B). Concentrations within this so-called "mutant selection window" (which also includes concentrations at which both sensitive and resistant virus have $R_0 > 1$, and $R_{resistant} > R_{sensitive}$, Rosenbloom et al. (2012)) select for resistance. The dynamics plotted in Figure

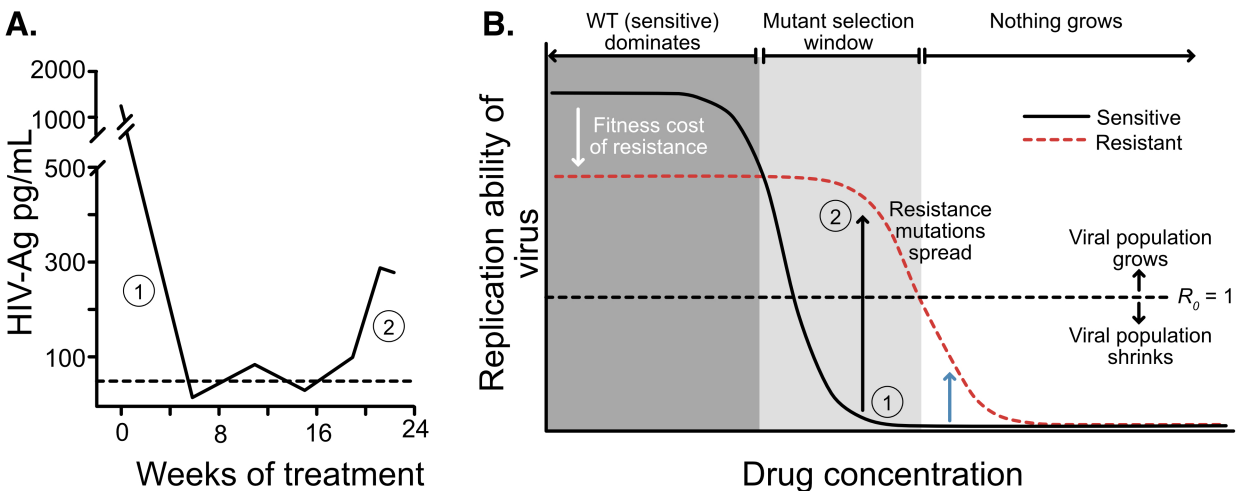

Figure 3: **A.** HIV antigen levels initially decline when treated with AZT monotherapy (6x200 mg/day) to below a detectable threshold (dotted line). Starting at week 16, the viral population begins to rebound even in the presence of the drug, indicating the evolution of drug resistance. Figure is replotted from Reiss et al. (1988). **B.** Schematic shows different regimes of viral behavior dependent on the concentration of a particular drug. At high concentrations (right side of the plot), both drug-resistant (dashed red) and drug sensitive (solid black) viruses are suppressed, with R_0 below 1. As drug concentration decreases, a window of concentrations emerges where the drug-resistant virus can grow in population size in the presence of the drug ($R_0 > 1$), but the drug sensitive genotype cannot. This is termed the "mutant selection window" (Drlica, 2003). At lowest concentrations, both wild-type (WT) and drug-resistant viruses can replicate, but the WT virus out-competes the drug-resistant type because it does not carry the fitness penalty of costly drug resistance mutations. Labels 1 and 2 show how the dynamics of the population in **A** can be explained by a mutation which behaves like the black arrow in **B**. If the resistance mutation only confers a change in R_0 according to the blue arrow, the viral population will remain suppressed.

3A suggest AZT concentration falls within this window: the population initially shrinks (Figure 3A, label 1) suggesting the drug-sensitive genotype must have $R_0 < 1$ (Figure 3B, label 1), but then begins to grow again (Figure 3A, label 2), suggesting that the drug-resistant genotype must be above the $R_0 = 1$ line (Figure 3B, label 2). In principle, had AZT dosage been higher (i.e., blue arrow in Figure 3B), the resistant virus would be unable to increase in size because its R_0 would be below 1 despite its resistance.

Combining three drugs that each bring the viral R_0 below 1, should result in an even lower R_0 (although how much lower is not straightforward, see Jilek et al. (2012)). Even if a virus acquires a resistance mutation rescuing its ability to replicate in the presence of one drug, as long as the combination of the three drugs suppresses R_0 below 1, that singly resistant virus should not spread. That we see partially resistant viruses reach detectable levels in patients means there must be a time or space where this virus replicates at $R_0 > 1$.

4.1 Temporal heterogeneity

After a patient takes a dose of treatment, the drugs are absorbed and metabolized within the body, resulting in an increase and then a decrease in drug concentration. Ideally, each drug is dosed such that the viral population cannot replicate under normal daily drug fluctuations (Figure 4A). Patient non-adherence and different rates of drug metabolism between patients can lead to drug concentrations that drop below the required levels over time. This has been widely recognized as a potential route to drug resistance (Fox et al., 2008; Bangsberg et al., 2007; Harrigan et al., 2005; Chesney et al., 2000; Cadosch et al., 2012).

The mutant selection window provides a useful lens through which to understand the consequences of missed doses. At drug administration, viral populations experience small R_0 values to the right of the mutant selection window and R_0 values increase as drug concentrations decay, moving to the left on the x-axis (Figure 4A). Drugs with short half-lives move quickly to lower concentrations, while drugs with long

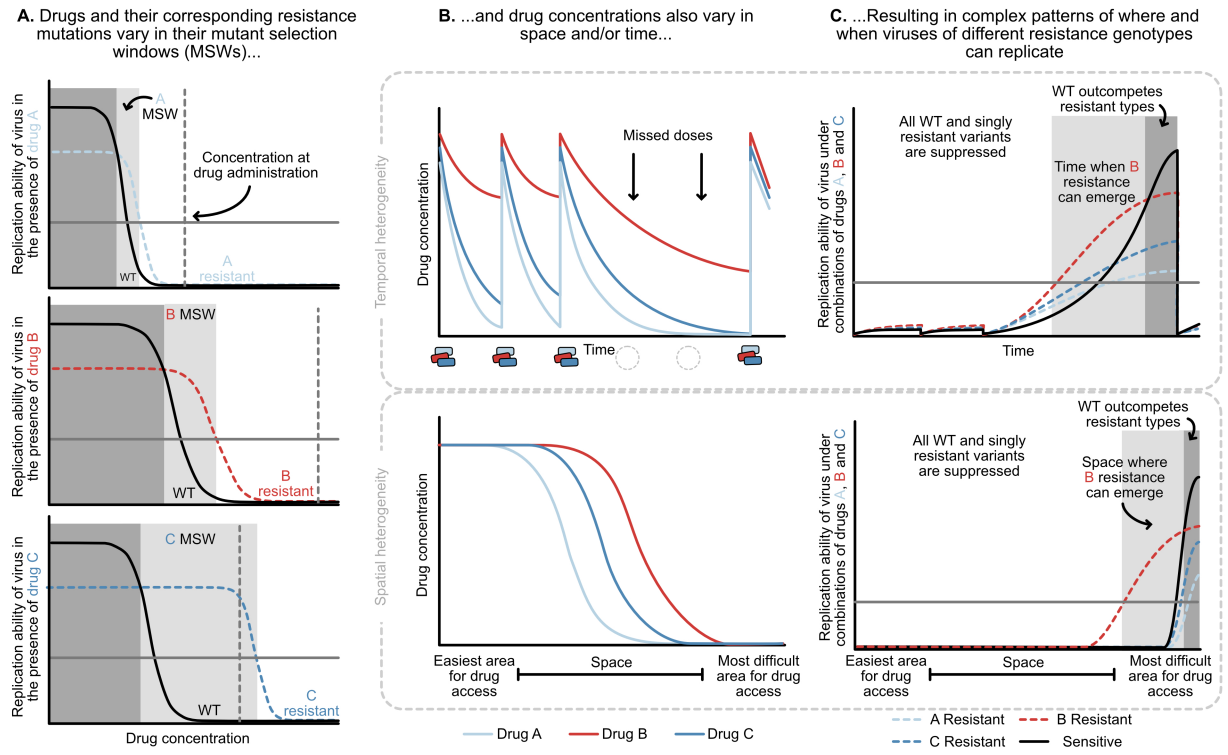

Figure 4: **A.** Different drugs and their associated drug resistance mutations have differently shaped concentration response curves and corresponding mutant selection windows (MSW). Pictured here, drug A (light blue) has the smallest MSW, whereas drug C (dark blue) has the widest MSW. Drug B (red) has an intermediate MSW. **B.** Variable MSWs also interact with drugs that have heterogeneous concentrations either in time (top) or space (bottom). Missed doses (due to incomplete adherence, plotted on top) can allow only drugs with long half-lives (here, Drug C) to remain present. This can create windows in time (pictured in **C.**) where resistance to only this longest half-life drug can be selected for. If drug levels are allowed to decline sufficiently low, viral replication of non-resistant types can emerge as well. Drugs that don't reach all of the body (plotted on bottom) can create areas where single drug resistance mutations (pictured in **C.**) can replicate. Areas where effectively no drugs reach can create a sanctuary where some viral replication of the wild-type (WT) virus can continue.

half-lives may move slowly through their mutant selection window. Missed doses may result in drug depletion of quickly decaying drugs and allow virus resistant to the longest half-life drug to replicate. Figures 4B and C show a scenario in which after missed doses, drug B in red with the longest half-life enters the mutant selection window after shorter half-life drugs leave the system. The dynamics clearly depend not just on drug B's mutant selection window, but also the rate at which all drugs are metabolized. To model this situation, it is therefore critical to incorporate the mutant selection windows, relevant drug interactions and drug concentration dynamics for all drugs.

In addition to understanding drug concentrations and the mutant selection windows, several other considerations are important. First, when the longest half-life drug enters its mutant selection window, are viruses with the right mutation present? Data from treatment-interruption studies suggests that short interruptions may not lead to resistance (De Truchis et al., 2017; Reynolds et al., 2010; Pennings, 2012), possibly because such mutations carry fitness costs and the population must grow substantially before it will produce them. Second, it is important to know under which conditions a resistant virus can remain present in the population, even as patients resume drugs intermittently. For the mutant to be successful, the decrease in virus while the patient is taking drugs must be smaller than the increase in virus during the mutant selection window. Patients missing doses too frequently may not select for resistant virus at all. It is unclear which adherence patterns result in this behavior, and if these same adherence patterns can explain why patients

fail treatment with resistance after 5-10 years on therapy after years of suppression.

We now return to the clinical data and ask whether these patterns may emerge from a model of temporal heterogeneity. Patients treated with 3TC+D4T+NFV acquire resistance largely in the order of 3TC, then NFV, then D4T. Both NFV and D4T are expected to lose most of their suppression within 24 hours due to short half-lives and, in the case of D4T, low potency (Rosenbloom et al., 2012). If a patient misses a dose, 3TC may quickly be the only potent drug present and enter its mutant selection window, allowing a 3TC-resistant genotype to spread. Indeed, we observe that 3TC mutations dominate over PI mutations among single drug resistance mutations in patients treated with PI therapy and an NRTI backbone containing 3TC (Figure 1A and 2C).

Unlike PIs, NNRTIs have particularly long half-lives (Taylor et al., 2007). Treatment interruptions of a few days are long enough for non-NNRTIs to leave the system, but short enough for NNRTIs to remain present. We would therefore expect NNRTI resistance to occur first, which is consistent with the data presented in Figures 1A and 2C. However, other data are not consistent with this explanation. An observational study conducted as part of the SMART trial on treatment interruptions tried to prevent a period of NNRTI monotherapy when interrupting treatment (so called staggered or switched stops) by "covering" the tail with additional shorter-lived drugs (Fox et al., 2008). It turned out that "covering" the tail did not affect the risk of drug resistance evolution (Fox et al., 2008), suggesting that the tail of NNRTI monotherapy may not be the reason NNRTI drug resistance evolves. We (Pennings) have proposed another explanation, namely that an increase in population size during an interruption allows resistance mutations to accumulate as standing genetic variation. When treatment resumes, these mutations increase in frequency due to selection (Pennings, 2012). Clearly, these observations demand more investigation into how temporal heterogeneity can generate such patterns, and indeed underscore the challenge of thinking about such observations without a quantitative model.

4.2 Spatial heterogeneity

HIV drugs do not penetrate throughout the body uniformly and penetration profiles differ among drugs. As a result, even in triple-drug-treated patients, parts of the body (e.g., brain, lymph nodes) may experience monotherapy (Else et al., 2011; Letendre et al., 2008; Decloedt et al., 2015; Fletcher et al., 2014; Thompson et al., 2019). Under a model of spatial heterogeneity, the concentrations that allow replication and expansion of drug-resistant virus (i.e., the mutant selection window of each drug) are distributed in physical space, as opposed to time. These concentration profiles may be largely overlapping and nesting (as illustrated in Figure 4B) or drugs may have semi-independent penetrance profiles, wherein some drugs reach certain parts of the body effectively, and other drugs reach other parts of the body effectively.

Different penetration profiles may result in parts of the body that consistently have drug concentrations that allow the replication of partially resistant genotypes (Figure 4C). A partially resistant virus migrating or reactivating from the latent reservoir in that part of the body may establish a growing HIV population. Even if these variants can only successfully replicate in one part of the body (i.e., the single drug compartment), they may still lead to a detectable viral load in a blood sample. After establishing in a single drug compartment, variants have the opportunity to acquire additional mutations that allow them to replicate elsewhere. In principle, this can explain the three observations above. First, drug resistance can continue to evolve at a constant rate as determined by the mutation rates and migration rates into such a single-drug compartment. Second, partially-resistant viruses may be observed if replication in one or more single drug compartments can be detected in the blood. Third, drugs with different penetrance profiles can explain why drug resistance evolves in a predictable order - namely in response to the best-penetrating drug first.

As in the temporal heterogeneity model, the spatial heterogeneity model may help us understand qualitative patterns previously observed in clinical trial data. In the Kempf study, patients were treated with 3TC, NFV, and D4T (see Figure 1A), where a plausible 3TC single-drug compartment may exist. Measurements of antiviral drugs in the central nervous system suggest that while 3TC and D4T successfully reach the central nervous system, NFV likely does not (Letendre et al., 2008; Browne et al., 1993; **?**). Fast decay of D4T may result in parts of the brain in which 3TC-resistant HIV can effectively replicate and spread, potentially allowing detection in the blood as a 3TC-resistant type. Once resistant to 3TC, the virus needs only one additional mutation to replicate in the presence of NFV. In practice, because of D4T's poor potency (Rosenbloom et al., 2012), much of the body may effectively be a 3TC+NFV compartment and select for

NFV mutations next (Fig 1A). Under a model of spatial heterogeneity, the probability of resistance emergence should depend on the sizes of the single drug compartment and the reservoir and the mutation and migration rates, which we would all expect to remain approximately constant over time.

Similar reasoning applies in the case of NNRTI-based therapies. NNRTIs tend to have good penetration profiles, which means that for NNRTI-based treatments, the spatial model may also explain why NNRTI resistance occurs first. However, many NNRTIs are highly suppressive even at relatively low concentrations and sometimes even for genotypes with resistance mutations. This suggests that an NNRTI resistance evolution may require not only a single drug compartment (i.e., the absence of other drugs), but also a sub-clinical concentration of NNRTI to select for resistance mutations.

For both the spatial and the temporal models there is an interesting and somewhat counter-intuitive effect. Intuitively, one might think that resistance will first arise in response to the "worst" drug in a cocktail (least potent, shortest half-life, or worst penetration profile). However, in a model of spatial or temporal heterogeneity, a desirable property –high penetration or long half-life– actually makes a drug vulnerable to resistance. Therefore, the best way to prevent the evolution of 3TC resistance (when treatment is with 3TC+NFV+D4T) is not to target the shortcomings of 3TC, but to target the shortcomings of the other drugs. Specifically, if 3TC monotherapy occurs in space or time because of a long half-life or good penetration, the solution would be to add another drug that also has a long half-life or good penetration.

The introduction of second generation PIs in the early 2000s resulted in exactly this intervention. In particular, ritonavir-"boosted" lopinavir (LPV/r) (Tseng et al., 2015) resulted in improved penetance and extended half-life in comparison to NFV (Capparelli et al., 2005). The clinical effect of replacing NFV with LPV/r in the previously described Kepmf study (Kempf et al., 2004) is a substantial reduction of resistance evolution by 76%. In the NFV arm of the study, 79 of 327 patients developed some type of drug resistance (24%) within 2 years, whereas in the LPV/r arm, only 19 of 326 did (6%).

In the context of the spatial model, LPV/r consistently reaches a level above the 50% inhibitory concentration in cerebrospinal fluid (CSF) (Capparelli et al., 2005), thereby likely shrinking the size or number of compartments in which 3TC exists alone. The fourfold lower rate of drug resistance evolution among patients on the LPV/r regimen suggests that the enhanced spatial coverage of LPV/r decreased the portions of the body where NFV reached alone by a factor of four. In the context of the temporal model, the longer half-life of LPV/r could reduce the times when 3TC was present alone. It should be possible to create models to test whether the difference in penetrance or the difference half-life better explain the reduced rate of resistance with LPV/r treatments.

5 Discussion

Above we have described ideas about how drug-level heterogeneity in time, due to different half-lives and non-adherence, can lead to periods of monotherapy, which, in principle, can lead to drug resistance evolution during triple-drug therapy. We have also described how heterogeneity in space, due to different penetration profiles of drugs, can lead to single-drug compartments, which also, in principle, can lead to drug resistance evolution. Therefore we have two possible explanations for the observation that drug resistance can evolve during triple-drug therapies. However, even if we know that temporal and spatial heterogeneity exist and *can* cause drug resistance evolution, this does not prove that they do.

Models that are intuitively convincing need not be true. For example, NNRTIs have long half-lives, which can lead to periods of monotherapy (the so-called "NNRTI tail") is patients miss doses or interrupt treatment. Additionally, HIV often evolves resistance to NNRTIs (Rocheleau et al., 2018). Yet, there is some evidence that the NNRTI tail does not explain NNRTI drug resistance in low-adherence patients (see discussion under temporal heterogeneity). A quantitative approach that includes mutant selection windows, pharmacokinetics and population genetics is needed to adjudicate between potential explanations. Can we build a model that can quantitatively match the observations?

Quite detailed models of HIV drug resistance have already been developed, especially for models of temporal heterogeneity. For example, in a carefully parameterized HIV model focused largely on single drugs (Rosenbloom et al., 2012), the authors examined how adherence patterns for a two-drug therapy (boosted darunivir and raltegravir, DRV/r + RAL) affected the mode and rate of resistance evolution. They found that virological failure could occur from a viral population resistant to raltegravir but not darunivir, because

of the characteristics of the darunivir mutant selection window. Similarly, Bershteyn and Eckhoff (2013) examined drug resistance dynamics in patients taking prep under a realistic model of patient non-adherence. However, these analyses of two-drug combinations served as proofs of concept, and no three-drug therapies were examined. A real gap therefore remains in developing realistic models that can explain complex patterns of resistance emergence under the most widely used triple-drug therapies.

For the spatial models, the gap between the models and clinical data is even larger than for the temporal models, because the models that have been studied are simpler (Perelson et al., 1997; Moreno-Gamez et al., 2015). Filling this gap will be particularly challenging because many parameters remain unknown. Understanding where, when, and how certain drugs reach different parts of the body is complicated (Vendel et al., 2019) and indeed, different studies have found different relative penetrances of drugs into the tissue versus the plasma. In addition, studies of drug penetrance may not be quantifying the correct factors to help us understand where drug resistance can emerge. First, the administered version of a drug may be at a different concentration than the active form (Dumond et al., 2008). Second, the intracellular concentration of a drug, rather than the tissue concentration, may mediate effectiveness, requiring that we understand not only whether drugs reach a tissue, but the rates at which they are transported into cells (Bazzoli et al., 2010; Dumond et al., 2008). Third, we may not be investigating penetrance in the correct compartments, or at the correct spatial scale. For example, there can be considerable heterogeneity of drug penetrance even within a single lymph node or gut cross section (Thompson et al., 2015, 2019). Fourth, many studies report tissue drug concentrations relative to the blood plasma, without considering the actual tissue drug level and its inhibitory effect on the virus (Else et al., 2011). Even comparing tissue drug concentration to the IC50 does not capture the full dynamics of drug concentrations within the body (Shen et al., 2008).

For both the spatial and temporal models, differences between people should also be taken into account. For example, treatment with other drugs (e.g., tuberculosis drugs) may affect the half-lives and penetration of HIV drugs (López-Cortés et al., 2002). Genetic variation among patients may also mediate penetrance (see Apostolova et al. (2015)). For example, efavirenz concentrations are higher in people with certain genotypes, and these genotypes are more common in Black/African groups. Rates of drug metabolism differ between men and women (Else et al., 2011). Different adherence patterns could hypothetically make different mutational orderings more or less likely as well. A recent paper suggests that the combination of imperfect adherence with imperfect penetration makes sequential drug resistance evolution likely in an in vitro system (Lustig et al., 2019).

For now, it remains unclear if temporal heterogeneity, spatial heterogeneity, or a combination of the two explain the evolution of drug resistance during triple-drug therapy. Clarity on this important unresolved mystery will aid the rational design of treatments that minimize the risk of resistance evolution.

Mathematical modeling has played an essential role throughout the HIV pandemic, and we do not intend to diminish these accomplishments. Early studies set a gold standard in uniting available measurements of viral populations (most often viral load) with quantitative models to understand many facets of HIV biology (see review in Nowak and May (2000)). Indeed, the reasoning within these studies helped motivate the need for triple-drug therapies in the first place (Perelson and Nelson, 1999). Now, armed with decades of genetic data, we need to continue this work to understand why triple-drug therapy is not evolution-proof.

Future studies addressing the evolution of multi-drug resistance in HIV should use realistic parameters (for example, like Rosenbloom et al. (2012) and Bershteyn and Eckhoff (2013)) and will need to grapple with the pharmacokinetics of multiple drugs and how they interact (Koizumi and Iwami, 2014; Jilek et al., 2012). We argue that these future studies must not just match viral loads and rates of patient failure, but also genetic patterns of resistance. In addition to the patterns described in this paper (ongoing at a near constant rate, sequential, and predictable resistance evolution), other observations should be considered: drug resistance rates have fallen over time (Feder et al., 2016), virologic failure varies as a function of patient adherence (Harrigan et al., 2005) and variable failure rates among different drug combinations (Lee et al., 2014; Feder et al., 2016). We hope that expanded models will allow us not only to make predictions - for example, about rates and patterns of resistance for novel combinations of drugs - but also to learn more about HIV's biology from data we already have. For example, can we use patterns of resistance to estimate how large single-drug compartments are, or how rapidly viruses migrate between different parts of the body (Feder et al., 2019)?

Drug resistance represents a serious threat across disease systems, and spatial and temporal variation in drug levels likely contributes in these other instances as well. For example, while many in the cancer com-

munity argue that applying multiple drugs simultaneously should halt the evolution of resistance (Basanta et al., 2011), experimental evidence also suggests multi-drug resistant cells may already exist as standing genetic variation (Hyo-eun et al., 2015). In treated solid tumors, drugs that cannot penetrate through the entire tumor may form a concentration gradient of selection pressure that theoretical (Hermsen et al., 2012; Fu et al., 2015; Gralka et al., 2017) and experimental (Baym et al., 2016) evidence suggests may speed the evolution of resistance. In tuberculosis, there is evidence that drug levels in various types of granulomas are often not as high as in the blood (and do not reach bactericidal levels) (Prideaux et al., 2015), and may be particularly low for some drugs in cavitary lesions (Strydom et al., 2019). Since not all tuberculosis patients have cavitary lesions, this means that ideal drug choice or dosing may not be the same for all patients (Strydom et al., 2019). Lessons about spatial and temporal heterogeneity in drug concentrations in HIV can form a scaffold upon which we can try to understand resistance evolution in other disease systems.

Even apart from disease, every natural system experiences multiple stressors simultaneously, even if those stressors are less extreme than targeted anti-retrovirals. How populations evolve in response to complex environments represents a fundamental question in evolutionary biology. In addition to (and perhaps because of) its medical relevance, HIV represents a powerful study system for interrogating this question. We understand many of its population parameters relating to viral kinetics. Extensive research has produced a well-developed genotype-phenotype map of resistance mutations. Because drug resistance emerges repeatedly in different patients, we can understand which features of this intra-patient evolution are generalizable, which provides insights into how evolution mediated by temporally and spatially varying selection pressures occurs.

In theory, multi-drug therapies should suppress pathogen populations and prevent the evolution of resistance. In practice, multi-drug therapies are challenging to achieve, and pathogen populations do not experience all drugs in all places at all times. When diagnosing the failure of any multi-drug approach because of resistance evolution (whether in cancer, antibiotics, herbicides, etc), we suggest the following four guiding questions: (1) Are all drugs effective? (2) Is there cross-resistance? (3) Is there spatial heterogeneity in drug levels? (4) Is there temporal heterogeneity in drug levels? Through understanding the ways that the application of multi-drug therapies fall short of the theory, we are poised to address those shortcomings and decelerate the evolution of resistance.

Methods

To determine the first mutation for a variety of triple-drug therapies, we analyzed data from Feder et al. (2016) representing Sanger-sequenced HIV populations treated with a broad range of regimens between 1989 and 2013. Each patient was treated with exactly one regimen to select against patients with pre-existing resistance before the onset of therapy. Ambiguous underlying nucleotide calls (i.e., non-A/T/C/G calls) were interpreted as population polymorphisms among all possible resulting amino acids. For example, an AAS residue (AAC/AAG) was recorded as an asparagine/lysine polymorphism, but an AAY (AAC/AAT) was recorded as asparagine. When multiple sequences were available for the same patient at the same time point, polymorphisms were also recorded.

We then determined the number of drug resistance mutations by comparing sequences to the WHO list of surveillance drug resistance mutations (DRMs) (Shafer et al., 2007). DRMs were only counted if HIV sequences contained residues conferring resistance to the therapy with which they were treated. Two classes of DRMs were recorded: 1) polymorphic DRMs in which some calls supported the DRM and some supported a non-DRM and 2) non-polymorphic DRMs, in which all calls supported the DRM.

To determine the first DRM, we retained only patients with exactly one polymorphic DRM or exactly one non-polymorphic DRM and any number of polymorphic DRMs (under the assumption that fixed mutations occurred before polymorphic mutations). For patients with sequences taken at multiple time points, we retained the first sample meeting the conditions above. In Figure 2B, we plot all three drug treatments with at least 25 patients. In Figure 2C, we plot all three drug treatments with at least 10 patients with a single drug resistance mutation.

Calculating target size and total mutation rate per drug

We based our calculations on the consensus subtype B sequence provided by the Los Alamos HIV Database (https://www.hiv.lanl.gov/content/index), the WHO list of resistance mutations (Shafer et al., 2007), and

mutation rates estimated in Abram et al. (2010). For each relevant amino acid position in protease and reverse transcriptase proteins, we determined all possible one-step mutations. For all one-step mutations, we determined whether they led to resistance to the relevant drugs according to the WHO list. We then summed the mutation probability for all possible one-step mutations that lead to resistance to a certain drug. The resulting total mutation probabilities ranged from $4 \cdot 10^{-5}$ for TDF to $2 \cdot 10^{-4}$ for LPV.

Plotting rates of drug resistance evolution over time

To create Figure 2A, we captured data from panels A and B of Figure 2 of Rocheleau et al. (2018) at three month intervals using WebPlotDigitizer (Rohatgi, 2020), and took a weighted average between the two time periods at each timepoint based on the sample sizes listed in the original paper ($N = 1441$ for 1996-2000 and $N = 1251$ for 2001-2005).

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