Review

# The impact of quasispecies dynamics on the use of therapeutics

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**The application of quasispecies theory to viral populations has boosted our understanding of how endogenous and exogenous features condition their adaptation. Mounting empirical evidence demonstrates that internal interactions within mutant spectra may cause unexpected responses to antiviral treatments. In this scenario, increased mutagenesis could be efficient at low mutagen doses due to the lethal action of defective genomes, whereas sequential administration of antiviral drugs might be superior to combination therapies. Our ability to predict the outcome of a particular therapy takes advantage of the complementary use of *in vivo* observations, *in vitro* experiments, and mathematical models.**

## Viruses as a spectrum of mutants

The quasispecies theory of molecular evolution mathematically describes the process of genome replication with production of error copies at the population level. It was initially proposed to explain self-organization and adaptability of primitive RNA or RNA-like genetic elements (also termed replicators or replicons) affected by error-prone template copying, and thus endowed with a huge population diversity [1–3]. This theory has exerted great influence in virology because of the observation that RNA viruses replicate in their hosts as complex mutant spectra, a population structure that resembles that of the primitive replicons as postulated by quasispecies theory ([4]; recent reviews in [5–7]). Formation of mutant spectra (also termed mutant distributions, swarms, or clouds; see Glossary) is fuelled by mutation rates of RNA genome replication in the range of $10^{-3}$ to $10^{-5}$ mutations introduced per nucleotide copied [8–10]. The molecular basis of high mutation rates lies in the absence in most RNA viruses of a proofreading–repair activity during RNA chain elongation [11,12], and in the inability of cellular post-replicative DNA repair enzymes to act on RNA [13].

Mutant swarms provide a broad repertoire of related but different genomes and phenotypes on which selection can act. The richness in variants has been repeatedly documented by biological and molecular cloning of viral genomes found in natural isolates, and recently by application of the new generation of ultra deep sequencing

## Glossary

**Combination therapy**: treatment that consists of the administration of two or more drugs.
**Complementation**: increase of viral progeny production mediated by gene products supplied by another virus (in quasispecies, supplied by closely related variants).
**Complexity of a mutant spectrum**: number of mutations and genomic sequences in a viral population. It is often quantified by pairwise genetic distances, mutation frequency (calculated by dividing the number of different mutations by the total number of nucleotides sequenced), and Shannon entropy (proportion of different genomes in the population). New technologies should allow a quantitative characterization of quasispecies complexity in terms of phenotypic diversity.
**Consensus sequence**: in a set of aligned nucleotide or amino acid sequences, the one that results from taking the most common residue at each position.
**Defective**: this term has several meanings. In viral populations, it may refer to mutant genomes unable to complete the viral cycle by themselves. Defectors are genomes that can replicate either on their own or under complementation, usually in the presence of the wild type. They can interfere actively with replication of the standard virus if the latter sequester nonfunctional or poorly functional *trans*-acting products expressed by the defectors. In the model of lethal defection, for instance, defectors have lost their ability to infect susceptible cells. In this review, both meanings are used and clarified when needed.
**Error threshold**: a theoretical average error rate that sets a maximum limit for maintenance of genetic information encoded by a replicating system. Error rates above the error threshold lead to loss of genetic information, also termed error catastrophe.
**Fitness**: when referred to in regard to viruses, fitness means the replicative capacity measured relative to some virus variant taken as reference. Fitness is environment-dependent.
**Interference**: this term has several meanings in biology. In the present review, it means the capacity of viral genomes to reduce the replicative activity of higher fitness genomes through *trans*-acting interactions. It can be regarded as the converse of complementation.
**Lethal mutagenesis**: viral extinction achieved through an excess of mutations, often promoted by mutagenic nucleotide analogs during viral genome replication.
**Master sequence**: the genomic nucleotide sequence that dominates a mutant spectrum because of its superior fitness. It may or may not be identical to the consensus sequence. The most abundant genome may still be a minority relative to the ensemble of low frequency variants. Owing to the abundance of quasineutral mutations and epistatic interactions in viral genomes, there might be a large ensemble of sequences of almost identical fitness that compose a 'master phenotype'.
**Monotherapy**: treatment that consists of the administration of a single drug.
**Mutant spectrum**: the ensemble of mutant genomes that compose a viral quasispecies. It is also termed mutant swarm or mutant cloud.
**Mutation frequency**: the proportion of mutated sites in a population of viral genomes. It is often calculated by dividing the number of different mutations found in a mutant spectrum by the total number of nucleotides sequenced.
**Mutation rate**: the frequency of occurrence of a mutation during viral genome replication.
**Rate of evolution**: the frequency of mutations that become dominant (i.e., are represented in the consensus sequence) as a function of time. It may refer to evolution within a host individual or upon epidemic expansion of a virus.
**Viral quasispecies**: a set of viral genomes that belongs to a replicative unit and subjected to genetic variation, competition, and selection, and which acts as a unit of selection. It has been extended to mean ensembles of similar viral genomes generated by a mutation–selection process.

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methodologies (reviewed in [7,14,15]). A detailed characterization of the phenotypic diversity of a quasispecies, instead, remains a major challenge. However, massive analyses which cross information from thousands of viral isolates of HIV [16] and barcode microarrays applied to polioviruses [17] constitute significant advances. Mutants with altered cell tropism or mutants that can escape the inhibitory action of drugs or antibodies are among those known to arise and populate mutant spectra at different frequencies. Such a phenotypic repertoire exposed to monotherapy (a single inhibitor of viral replication) could select for drug-resistant mutants and synthetic vaccines that expose the host to a limited number of epitopes (as compared with the complete virus) are unlikely to produce solid protection [7,18–23]. Clearly, combination therapies and multi-epitopic vaccines are needed to counteract adaptability ensuing from mutant spectrum complexity and quasispecies dynamics, although these recommendations are not universally followed.

Despite the remarkable success of antiviral combination therapies, the reality in clinical practice is that selection of multidrug-resistant viral mutants is still a frequent cause of viral breakthrough and treatment failure. The problem is more severe for viruses that produce some type of genomic reservoir as part of their life cycle. This is the case for the proviral DNA in the replication cycle of retroviruses such as HIV-1 or the circular covalently closed DNA of hepatitis B virus (HBV) (a virus that despite having a DNA genome uses RNA as replicative intermediate, with an error-prone reverse transcription step) (reviews in [7,24]). Despite the essential Darwinian features of quasispecies dynamics (generation of mutants, competition among them, and advantage of fitter variants) being common to all RNA viruses, a genetic reservoir of the type present during HIV-1 and HBV infections provides a repository of genomic sequences (quasispecies memory) that may contribute to the reintroduction of escape mutants into the replicating pool (reviewed in [7,25]). In addition, viral infective strategies (as degree of virulence or replication mode) and environmental variables (e.g., frequent population bottlenecks) play important roles in the collective response of viral populations.

Antiviral therapy has to consider not only the phenotypic profile of mutant distributions, possible adaptive strategies, molecular memory, and exogenous variables but also a feature of viral quasispecies not suspected a decade ago: internal interactions within mutant spectra (Figure 1). This review deals with the dynamics of RNA virus replication in the presence of an antiviral inhibitor and a mutagenic agent, and its consequences for optimal antiviral treatment. In particular, parameter values that should favor the efficacy of a sequential (inhibitor–mutagen) versus the corresponding combination administration are presented.

## Complementation and defection as a collective property of viral quasispecies

The initial quasispecies theory emphasized that the quasispecies as a whole, rather than individual genetic elements, was the true target of selection [3]. Within a quasispecies, genomes are mutationally coupled, such that the abundance of a given variant is not only a function of its replicative ability in isolation but also depends on how often it is produced through mutation of neighboring genotypes. For many years, the implications of a quasispecies organization were not tested with viruses, fundamentally because of the lack of suitable experimental designs. Viral quasispecies were regarded as the result of a mutation–selection balance as proposed by classical population genetics [26], sometimes referred to as the Wright–Fisher formulation. However, it is now well established experimentally and through theoretical models that interference and complementation can occur within mutant spectra and influence viral performance (reviewed in [7]). In a given environment, the outcome of an antiviral treatment depends on the organization of the viral population and on its momentary genomic composition, as well as on the internal interactions within viral subpopulations. Antiviral treatments are often decided on the basis of the available drugs, tolerability of side effects, and efficacy in decreasing viral load. Yet, it is increasingly recognized that rarely the joint activity of two or more drugs can be reduced to the addition of their independent effects [27–29]. Next, we briefly describe alternative antiviral designs, and how mutant spectra can influence the efficacy of treatments in which a mutagenic agent is involved.

## Intra-mutant spectrum interactions and virus extinction

The advantage of combination therapies over monotherapy to control virus replication has been amply supported by clinical, experimental, and theoretical studies [7,18–21,30–34]. Additional designs based on other forms of drug and immunotherapeutical combined approaches have been proposed [35–42] but few of them have become standard clinical practice.

The possibility to eliminate infectious virus using mutagenic agents was inspired by the concept of error threshold derived from quasispecies theory that asserts that there is a maximum error rate (termed the error threshold) compatible with maintenance of genetic information [3,43,44]. Use of virus-specific mutagenic agents to control viral infections is now known as lethal mutagenesis. An adverse effect of mutagenic agents on infectious progeny production has been validated with a variety of RNA viruses that display different replication mechanisms ([45–58]; reviewed in [7,43,59–63]).

The term ‘error catastrophe’ was coined by Leslie Orgel to refer to the negative effects of mistakes during the process of protein synthesis, in relation to aging [64,65]. In quasispecies theory, error catastrophe means the transition towards loss of superiority of the master sequence brought about by an excess of mutations relative to the maximum compatible with maintenance of the genetic information [3,43]. In the case of RNA viruses, the mechanisms by which an excess of mutations leads to loss of infectivity must be different from those postulated to occur during the transition to error catastrophe. Viral populations include multiple viable variants with different fitness values, and loss of the fittest (which can be regarded as the equivalent to loss of superiority of the master sequence) need not imply elimination of other components of the mutant spectrum [66]. Actually, the increase of viral

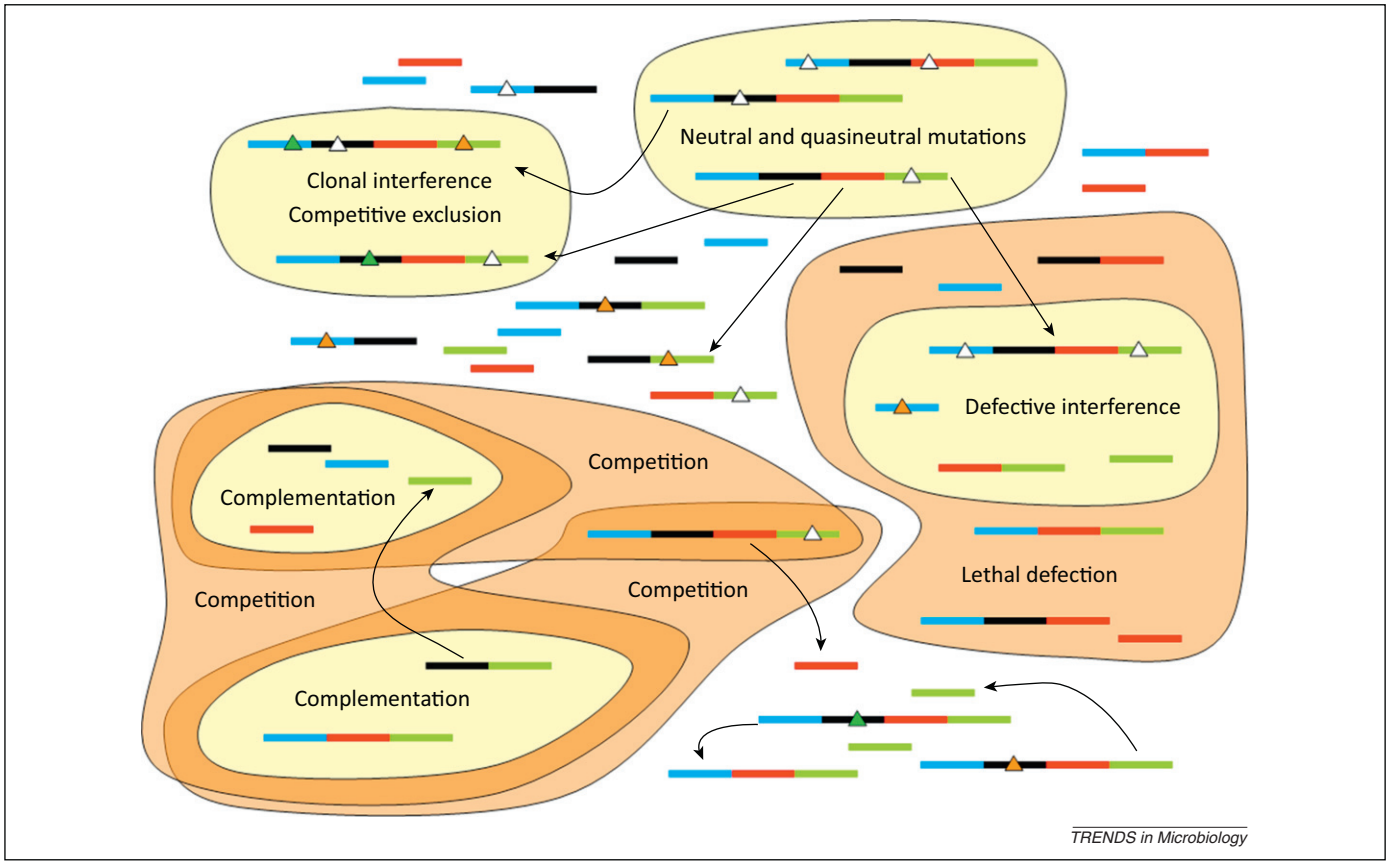


**Figure 1**. Schematic representation of some of the interactions within mutant spectra. Viral quasispecies are vast ensembles of different genomes. Under replication, genomes (represented by colored lines) suffer different types of mutations. Here, triangles denote point mutations and their color for their value: neutral or quasineutral (white), deleterious (orange), or beneficial (green). The deletion of a whole genome segment produces defective (incomplete) genomes. Neutral mutations allow a costless exploration of genome space. The structure of the network of neutral mutations, which can be huge, conditions the genomic composition of the quasispecies and its adaptability [80]. Mutational coupling between genomes is illustrated through thin black lines. A whole genealogy of a quasispecies could be traced, in which case every genome in the picture would either receive or send at least one arrow. When two replicating genomes are hit by independent beneficial mutations, clonal interference occurs. This type of interference impedes the fixation of beneficial mutations and delays their spread to the population. Eventually, a fraction of beneficial mutations is lost due to competitive exclusion. The production of defective genomes always represents a burden to the population. An excess of a particular type of defective genomes may cause the extinction of the whole population (lethal defection, Box 1). Another type of interaction between defective genomes is complementation, provided by any genome codifying for gene products absent in the defector: two examples are shown in the figure. This leads, at a higher hierarchical level, to competition between different subgroups (formed by different combinations of defective genomes) and with the wild type (Box 1). All these interactions, and probably others, take place simultaneously among the genomes that form the quasispecies.

diversity entailed by increased mutagenesis could favor survival of poorly adapted viral quasispecies [67], causing an effect contrary to that expected. Processes absent in ensembles of simple replicators may be acting in viral populations: predicting the effect of an antiviral drug in a general situation is a difficult task, and even educated guesses fail, as in the next example.

A study of the consequences of 5-fluorouracil (FU) mutagenesis on lymphocytic choriomeningitis virus (LCMV) during a persistent infection in cell culture showed that, unexpectedly, the decay of infectivity preceded the decay of viral RNA. The delayed decrease of LCMV RNA relative to infectious virus suggested that a class of noninfectious, but replication-competent LCMV RNAs were present in the transition towards extinction [56]. As with many other RNA viruses, defective genomes are produced during LCMV replication. Because no LCMV infection can be established without generation of defective mutants, a computational model was developed to predict the fate of LCMV under different mutagenic intensities and in the absence or presence of defective (noninfective) genomes [56]. Two extinction pathways were found. At low mutational intensities, extinction was dependent on a class of defective genomes in which infectivity was lost earlier than replicative ability: viral RNA did not decrease but infectivity did, as observed experimentally (Box 1). We term this class of defective genomes defectors. They are characterized by being competent in RNA replication (on their own or under complementation) but endowed with the capacity to interfere with replication of the standard, infectious genomes. Interference may result from expression of functionally impaired proteins that form complexes with other proteins, resulting in diminished biological activity relative to complexes composed of fully functional proteins [7,56,68]. At high mutagenic intensities there was no effect of defectors, and both infectivity and viral RNA were simultaneously lost. Additional studies, both theoretical and experimental, have supported lethal defection as a mechanism of virus extinction [7,66,69].

At present, we view virus extinction by mutagenic agents as a complex process in which the mutagen increases the frequency of defectors that gradually convert a quasispecies dominated by complementation into a quasispecies dominated by interference. The result of a

**Box 1. Lethal defection and complementation**

An artificial increase in the error rate of viral replication through the use of mutagenic drugs is an efficient mechanism to cause the extinction of viral infectivity *in vitro*. However, it is still unclear how the extinction transition proceeds: the response of a viral population to the action of a mutagen is not a simple function of the mutagen dose.

Mutagenic drugs enhance the appearance of defective genomes and reinforce genetic drift. In persistent infections, fluctuations in the relative population numbers (e.g., of infective vs noninfective genomes) may cause the extinction of the population in short times at relatively low values of the mutation rate [56]. If the multiplicity of infection is low, defective (incomplete) genomes are also eliminated with every new infection, because they cannot complete the replicative cycle in isolation [66]. However, replication-competent, low fitness (or suboptimal) genomes can be present (and can infect or be generated intracellularly) and interfere with replication of high fitness genomes [75]. The outcome of the infective process is different at high multiplicity of infection, where defectors which have maintained their infective capacity may be complemented – also by other defective forms, and lead to the emergence of cooperative groups of incomplete genomes that become viable in the absence of the wild type (Figure I). In situations in which a shorter genome confers some adaptive advantage, the combination of complementation and competition may cause the extinction of the wild type [77], offering a plausible explanation for the origin of bipartite viruses [78]. A possible control mechanism for multipartite virus could be forcing their transmission at low multiplicity of infection (MOI, or the number of infectious units per target cell), thus precluding complementation and replication.

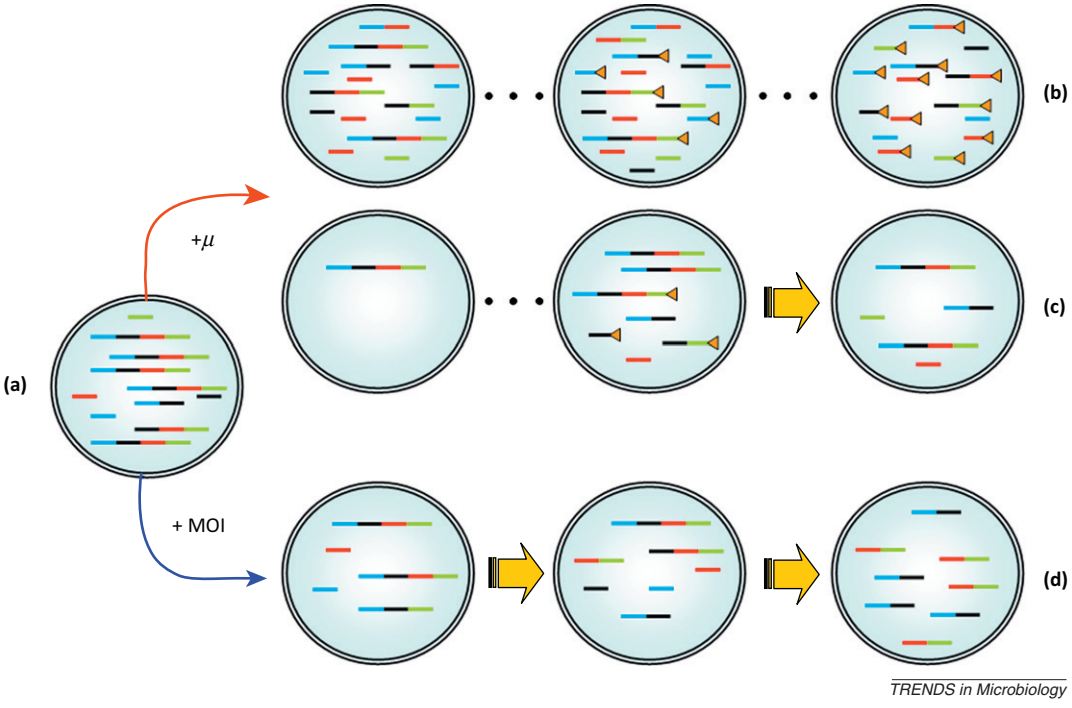


**Figure I.** Viral dynamics depend on survival strategies, environmental variables, and selective pressures. **(a)** Inside a cell, genomes of various types coexist, represented by various colors. The infective and replicative strategy of a virus guarantees its survival in its natural environment. When the mutation rate ($+\mu$) is slightly increased through the use of mutagenic drugs (indicated by the red arrow), survival depends on the infective strategy. **(b)** In persistent infections, intracellular viral genomes compete for replication and noninfective variants are accumulated (genomes with an orange triangle). This mechanism, which may usher in extinction, defines lethal defection. Points between cells link the same cell at different times. **(c)** In lytic infections, noninfective variants are cleared up every time a new susceptible cell is infected (indicated by orange arrows) and, under the same mutagenic dose, the population may survive longer. **(d)** If the natural multiplicity of infection of a virus is increased (blue arrow, +MOI), and shorter genomes enjoy a large enough advantage (higher stability or faster replication, for instance), a cooperative group of complementary, defective forms may displace the wild type. These different situations indicate that the effects of a drug vary depending on the infective strategy of a virus, among other influences.

sustained mutagenic activity is the precipitous collapse of viral functions that results in extinction. This tentative scenario is well supported by theory and experiments, and it is under further investigation. Importantly, the participation of defectors in virus extinction can be of relevance for the design of antiviral protocols based on lethal mutagenesis.

## Sequential versus combination therapy: a disadvantage of togetherness

Although the objective of treatment protocols is obviously to eliminate a virus from infected patients, preliminary experiments in cell culture and then in animal models are needed to explore the efficacy of new drugs and drug combinations, prior to considering a possible clinical application. This is particularly true with antiviral treatments based on administration of mutagenic agents. Only one clinical trial using this design has been performed to date and this involved administering a mutagenic pyrimidine analog in monotherapy to AIDS patients [70].

In agreement with the recognized advantages of combination therapy, it was observed that high fitness viruses were more efficiently extinguished with a combination of a mutagenic agent and an antiviral inhibitor than with a mutagenic agent alone. This was shown with foot-and-mouth disease virus (FMDV) [54] and HIV-1 [55]. The effectiveness of a combination of an inhibitor and a mutagenic agent cannot be interpreted merely as the sum

**Box 2. Modeling sequential versus combined therapy protocols**

Experimental results demonstrate that the action of a mutagenic drug decreases the viral yield in a fixed percent at each passage, the decrease depending on the administered dose [74]. The response to the action of an inhibitor is different due to the likely appearance of resistant forms: the variation in yield depends on the dose but also on the population size [79]. In the presence of an inhibitor, the viral yield results from the addition of two terms: one representing the decrease in replicative ability of the susceptible population and a second one standing in for the replication of possible resistant forms. Whereas the first term implies a decrease in yield at initial passages, the second term becomes dominant as passages elapse. As a result, the yield as a function of time is U-shaped for any dose of inhibitor that does not cause extinction at early times. The reduction in yield caused by the mutagen or by the inhibitor at initial passages can be experimentally quantified by comparing the viral yield obtained with and without either drug.

In this scenario, there are two relevant types of mutations: those turning the virus into a defective mutant and those conferring resistance to the inhibitor. The former appear at a rate $\omega$, and the latter at a rate $\mu$. The ratio $\omega/\mu$ is independent of the global mutation rate, and $\mu$ is much smaller than $\omega$. Assume that, initially, all viral genomes are susceptible to the inhibitor. Let us call $s(g)$ and $r(g)$ the number of susceptible and resistant genomes present at the intracellular replication cycle $g$. With the rules above, their numbers vary as:

$$s(g+1) = i\,(1-\mu-\omega)\,m\,s(g)$$

$$r(g+1) = i\,\mu\,m\,s(g) + (1-\omega)\,m\,r(g)$$

where $m$ is the total number of copies produced per genome and replication cycle and $i < 1$ represents the decrease in the viral replicative ability due to the inhibitor. One passage consists of $G$ replication cycles, this parameter, and $m$ depending on the replicative strategy of the infecting virus. When defectors are cleared up at each passage, they do not have a relevant effect in the dynamics, and hence their dynamical equations can be dismissed (Figure I).

The application of the combination treatment requires calculating the viral yield as the sum of susceptible and resistant forms after $G$ cycles. To mimic the sequential treatment, we model a first passage ($G$ cycles) with $\omega = \omega_0$ (obtained from the basal mutation rate of the virus, no mutagen) and a second passage that begins with the population just generated, and has $i = 1$ (no inhibitor) [29]. These rules can be easily generalized to represent situations where two mutagens or two inhibitors are used instead.

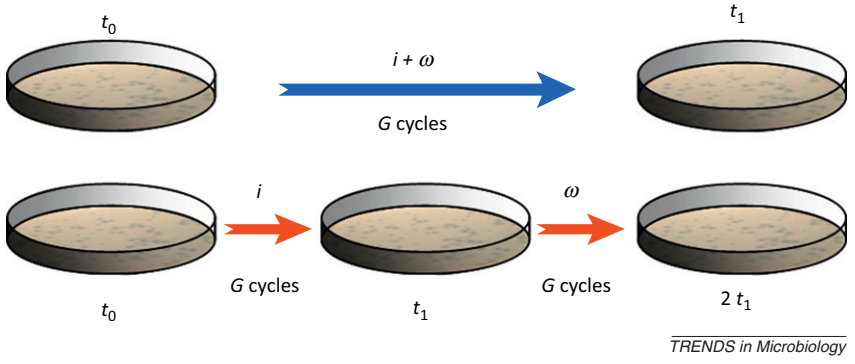


**Figure I.** Cartoon of the experimental protocol. In the *in vitro* assays of combination therapy, a dose of inhibitor (with effect implemented as $i$ in the model) and a dose of mutagen (implemented as $\omega$) are simultaneously administered (above). In sequential therapy, they are dispensed one after another (below). Note that, in either treatment, the time of action of any of the two drugs is the same ($t_1$), corresponding to $G$ intracellular replication cycles. Adapted from [29].

decrease of viral load that would be produced by the inhibitor and the mutagen administered independently. Owing to the nontrivial interaction between the two drugs, the probability of appearance and selection of escape mutants resistant to the inhibitor depends on the presence of inhibitors of viral replication, but can be enhanced through the use of mutagenic agents, which themselves can also select for mutagen-resistant mutants [51,71–73].

A comparative study with FMDV showed that a sequential administration of the inhibitor of the RNA replication guanidine, followed by the mutagenic nucleoside analog ribavirin, resulted in a more effective viral extinction than the corresponding combination treatment (simultaneous administration of guanidine and ribavirin) [74]. The advantage of the sequential treatment was more pronounced when the initial concentration and inhibitory activity of guanidine were high. At least two factors have been identified as being probably involved in the sequential inhibitor–mutagen administration being more effective. The first factor is the effect of the interaction between a mutagen and an inhibitor of viral replication with the components of the target mutant spectrum (Box 2). The second factor is the requirement of defector genomes to be RNA replication competent in order to exert their interfering action. A correlation between positive RNA replication and interfering activity was demonstrated with several capsid and polymerase FMDV mutants, including a double polymerase mutant which lost its interfering activity when a third mutation that rendered the mutant RNA replication negative was added to the viral genome [75]. The presence of an inhibitor of viral RNA replication impeded the interference by specific FMDV mutants [74]. Therefore, the simultaneous presence of an inhibitor and a mutagen can jeopardize a decrease of replicative capacity and viral load mediated by defectors [56,74].

The observation that a sequential administration of two drugs could have an advantage over the corresponding combination was unexpected and encouraging. It meant that when a mutagenic agent participates in therapy, some of the side effects derived from combination treatments could be avoided. However, the possible recommendation of a sequential protocol requires careful examination of the range of parameters under which it displays an advantage over combination treatment.

### Analysis of multidrug therapies through computational models

The appropriateness of sequential versus combined protocols involving two antiviral drugs can be systematically explored by means of simple models that take into account the action mechanism of each drug. Such models are especially appealing as a guide to the design of preliminary *in vitro* assays, where the absence of the immune system and structural tissue complexities improves their predictive ability.

In experiments comparing sequential versus combination therapies, a very simple model can be derived if several conditions hold. First, replication mechanisms do not include provirus phases (as retroviruses) or latency steps (as herpesviruses), which would require a careful evaluation of time delays. Second, the viral load decreases when the therapy is applied, so that competition for resources relaxes, and resource limitation does not need to be considered in the model. Third, the multiplicity of infection is low, which might result in diminished complementation or interference by defective genomes.

Under such conditions, intracellular viral dynamics can be modeled as a series of genome replicative cycles, with each cycle resulting in several copies made from the templates obtained in the previous one. Thus, the exact meaning of a replicative cycle depends on the replication mechanism of the virus: for single-stranded RNA (ssRNA) genomes, the replicative cycle refers to the synthesis of multiple genomic strands from each complementary strand; whereas for double-stranded DNA (dsDNA) genomes with semiconservative replication, the replicative cycle is just the semiconservative replication itself. The hypothesis above, together with the known action of the two antiviral drugs, can be synthesized in a few dynamical equations that allow predicting the response of the viral population to different protocols and drug doses (see an example in Box 2).

Straight modifications of the model described in Box 2 allow for an analogous evaluation under different drug protocols. As a first result, therapies involving two similar drugs (two inhibitors or two mutagens) are more efficient when administered in a combined way. However, if an inhibitor and a mutagen are used, the sequential protocol may be preferable depending on drug doses and clinical criteria (maximal reduction of viral titer versus prevention of viral resistance), as schematically depicted in Figure 2. The root of this dose-dependent behavior lies at the double role that mutagens play. The exposure of the virus to mutagenic drugs increases the mutation rate. At low multiplicities of infection, no complementation takes place and defective mutants behave as lethal, so an increase in the mutation rate leads to a reduction in the production of viable viruses. However, at the same time, the increase in the mutation rate accelerates the appearance of mutants that are resistant to the inhibitor, thus leading to a nonlinear interaction between the two drugs that could yield unwanted effects.

According to Figure 2, the optimal protocol for the administration of an inhibitor and a mutagen depends on drug doses. In addition, the dose combinations for which a sequential or a combined protocol is preferred vary depending on biological properties of the virus. This means that for different viruses the drug doses that make the sequential therapy more effective (red regions in Figure 2) may change. In practice, a given protocol is suitable if it becomes advantageous for a wide range of drug combinations. In the particular case of a sequential inhibitor–mutagen protocol, it is expected to be more suitable when applied to viruses with a small to moderate yield and a replication mechanism that produces many copies from the same template (e.g., ssRNA viruses with replication via minus strands that each produce many plus strand RNA copies).

Although combination therapies of similar drugs are more effective at clearing infections than individual administration, a synergistic interaction between the two drugs may be present. Under conditions of strong

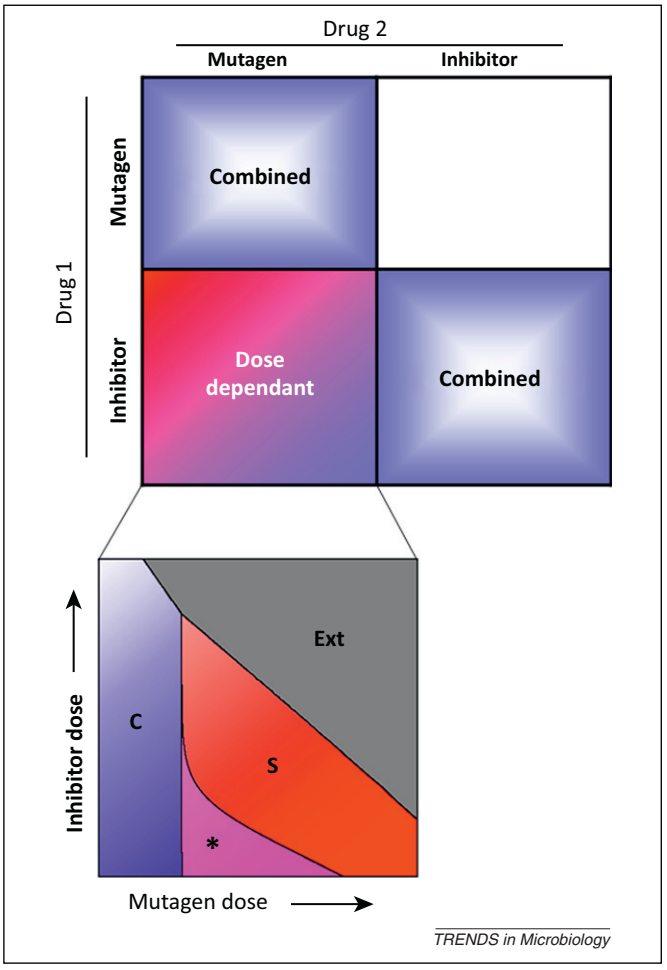


**Figure 2**. Optimal protocol choice in multidrug therapies. According to theoretical models, the optimal protocol for drug administration in multidrug therapies depends on the action mechanism of the drugs. If two inhibitors (or two mutagens) are to be used, their efficiency is optimized through simultaneous administration (combined protocol). However, for mixed inhibitor–mutagen therapies the optimal protocol depends on the nature of the virus and the drug doses: the lower panel shows this dependence for foot-and-mouth disease virus (FMDV) with guanidine as inhibitor and ribavirin as mutagen [29]. Blue region (C): combined protocol performs better than sequential administration; red region (S): sequential protocol – first inhibitor, second mutagen – performs the best; gray region (Ext): at high drug doses, the virus becomes easily extinct with both protocols; pink region (*): combined protocol is more effective in reducing the viral titer but it produces resistant mutants with higher probability than the sequential protocol. The sequential protocol when the mutagen is provided before the inhibitor is not considered because its performance is always worse than the others.

competition for resources, multidrug resistant mutants may encounter a selective advantage higher than when the two drugs are independently dispensed. There is a critical level of synergistic interaction between drugs above which the advantage of combined administration is suppressed by the increased risk of multidrug resistance [27]. This is another instance of combination therapy not being in all cases superior to sequential monotherapies, once more due to the interaction between drugs and the nontrivial response of the population.

At present, mathematical models aimed at yielding specific predictions need to be formulated in conjunction with empirical results and should focus on a single or few observations to improve their predicting power [76]. Models tailored to a particular population and environment necessarily suffer from restricted applicability and should only be applied to other experimental systems (a different virus, for instance) once the specific features of the new system have been formally taken into account.

The use of mathematical models analogous to those reviewed here can significantly reduce the number of *in vitro* assays to be performed in infections caused by viruses using various replicating strategies. This variation appears in general easy to translate into dynamical equations similar to those in Box 2. The simulation of an *in vivo* situation certainly entails additional difficulties such as the interaction with the immune system, or environmental and individual characteristics. The predictions of any model, once tested *in vitro*, should be taken only as a rough guide to apply one or another administration protocol and to infer minimum drug doses in *in vivo* assays.

## Concluding remarks

In conclusion, the internal interactions among components of a mutant spectrum render it such that a combination treatment is not always the preferred protocol to suppress viral replication. There is sufficient theoretical and experimental evidence *in vitro* to strongly suggest the convenience of using a sequential therapy when the two drugs interact in a manner analogous to that described for a mutagen and an inhibitor. To avoid the appearance of resistant forms, it is advisable that mutagens are used alone or when the viral load is as low as possible. When either two inhibitors or two mutagens are used, our model predicts that a combination treatment is always preferred over a sequential treatment. The least effective strategy is predicted to be sequential mutagen–inhibitor administration. Extensions of the results with viruses in cell culture to animal models are needed to evaluate alternative antiviral protocols to be applicable to complex environments (Box 3).

### Box 3. Outstanding questions

- New mutagenic agents specific for viral polymerases should be developed based on an increasing knowledge of polymerase structure and fidelity determinants.
- *In vivo* tests to compare reduction of viral load as a result of the sequential versus combined administration of one or several inhibitors and one or several mutagens are needed. Ideally, they should include the use of animal models and clinical trials with patients that have lost other therapeutic options.
- Suitable animal models include persistent arenavirus infections in mice and hepatitis C virus infections of mice with a liver repopulated with human cells. A clarification of the mutagenic activity of ribavirin in these systems is needed.
- Extensions of theoretical models to contemplate internal interfering interactions within quasispecies and participation of multiple inhibitors and mutagens (displaying the same or different mutagenic specificity) should be developed, and theoretical predictions contrasted with experimental results.
- Simulations that capture some environmental complexities during viral infections are desirable. Such simulations may not lead to deterministic equations but may have to incorporate fluctuating dynamic variables.

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### References

1 Eigen, M. (1971) Self-organization of matter and the evolution of biological macromolecules. *Naturwissenschaften* 58, 465–523
2 Eigen, M. (1992) *Steps Towards Life*, Oxford University Press
3 Eigen, M. and Schuster, P. (1979) *The Hypercycle. A Principle of Natural Self-organization*, Springer
4 Domingo, E. *et al.* (1978) Nucleotide sequence heterogeneity of an RNA phage population. *Cell* 13, 735–744
5 Domingo, E. (2006) *Quasispecies: Concepts and Implications for Virology (Current Topics in Microbiology and Immunology, Vol. 299)*, Springer
6 Lauring, A.S. and Andino, R. (2010) Quasispecies theory and the behavior of RNA viruses. *PLoS Pathog.* 6, e1001005
7 Domingo, E. *et al.* (2012) Viral quasispecies evolution. *Microbiol. Mol. Biol. Rev.* 76, 159–216
8 Batschelet, E. *et al.* (1976) The proportion of revertant and mutant phage in a growing population, as a function of mutation and growth rate. *Gene* 1, 27–32
9 Drake, J.W. and Holland, J.J. (1999) Mutation rates among RNA viruses. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13910–13913
10 Sanjuan, R. *et al.* (2010) Viral mutation rates. *J. Virol.* 84, 9733–9748
11 Steinhauer, D.A. *et al.* (1992) Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. *Gene* 122, 281–288
12 Ferrer-Orta, C. *et al.* (2006) A comparison of viral RNA-dependent RNA polymerases. *Curr. Opin. Struct. Biol.* 16, 27–34
13 Friedberg, E.C. *et al.* (2006) *DNA Repair and Mutagenesis*, American Society for Microbiology
14 Vandenbroucke, I. *et al.* (2011) Minor variant detection in amplicons using 454 massive parallel pyrosequencing: experiences and considerations for successful applications. *Biotechniques* 51, 167–177
15 Macalalad, A.R. *et al.* (2012) Highly sensitive and specific detection of rare variants in mixed viral populations from massively parallel sequence data. *PLoS Comput. Biol.* 8, e1002417
16 Hinkley, T. *et al.* (2011) A systems analysis of mutational effects in HIV-1 protease and reverse transcriptase. *Nat. Genet.* 43, 487–489
17 Lauring, A.S. and Andino, R. (2011) Exploring the fitness landscape of an RNA virus by using a universal barcode microarray. *J. Virol.* 85, 3780–3791
18 Bonhoeffer, S. *et al.* (1997) Virus dynamics and drug therapy. *Proc. Natl. Acad. Sci. U.S.A.* 94, 6971–6976
19 Müller, V. and Bonhoeffer, S. (2008) Intra-host dynamics and evolution of HIV infections. In *Origin and Evolution of Viruses* (2nd edn) (Domingo, E. *et al.*, eds), pp. 279–302, Elsevier
20 Domingo, E. (1989) RNA virus evolution and the control of viral disease. *Prog. Drug Res.* 33, 93–133
21 Domingo, E. and Holland, J.J. (1992) Complications of RNA heterogeneity for the engineering of virus vaccines and antiviral agents. *Genet. Eng. (N. Y.)* 14, 13–31

22 Taboga, O. *et al.* (1997) A large-scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of escape mutants. *J. Virol.* 71, 2606–2614
23 Zubkova, I. *et al.* (2009) T-cell vaccines that elicit effective immune responses against HCV in chimpanzees may create greater immune pressure for viral mutation. *Vaccine* 27, 2594–2602
24 Quer, J. *et al.* (2008) The impact of rapid evolution of hepatitis viruses. In *Origin and Evolution of Viruses* (2nd edn) (Domingo, E. *et al.*, eds), pp. 303–350, Elsevier
25 Briones, C. and Domingo, E. (2008) Minority report: hidden memory genomes in HIV-1 quasispecies and possible clinical implications. *AIDS Rev.* 10, 93–109
26 Wilke, C.O. (2005) Quasispecies theory in the context of population genetics. *BMC Evol. Biol.* 5, 44
27 Torella, J.P. *et al.* (2010) Optimal drug synergy in antimicrobial treatments. *PLoS Comput. Biol.* 6, e1000796
28 Fitzgerald, J.B. *et al.* (2006) Systems biology and combination therapy in the quest for clinical efficacy. *Nat. Chem. Biol.* 2, 458–466
29 Iranzo, J. *et al.* (2011) Tempo and mode of inhibitor–mutagen antiviral therapies: a multidisciplinary approach. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16008–16013
30 Ho, D.D. (1995) Time to hit HIV, early and hard. *N. Engl. J. Med.* 333, 450–451
31 Le Moing, V. *et al.* (2002) Predictors of virological rebound in HIV-1-infected patients initiating a protease inhibitor-containing regimen. *AIDS* 16, 21–29
32 Nijhuis, M. *et al.* (2009) Antiviral resistance and impact on viral replication capacity: evolution of viruses under antiviral pressure occurs in three phases. *Handb. Exp. Pharmacol.* 189, 299–320
33 Pol, S. *et al.* (1999) A randomized trial of ribavirin and interferon-α vs. interferon-α alone in patients with chronic hepatitis C who were non-responders to a previous treatment. Multicenter Study Group under the coordination of the Necker Hospital, Paris, France. *J. Hepatol.* 31, 1–7
34 Van Vaerenbergh, K. *et al.* (2002) Initiation of HAART in drug-naive HIV type 1 patients prevents viral breakthrough for a median period of 35.5 months in 60% of the patients. *AIDS Res. Hum. Retroviruses* 18, 419–426
35 Li, M.J. *et al.* (2005) Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol. Ther.* 12, 900–909
36 Seiler, P. *et al.* (2000) Additive effect of neutralizing antibody and antiviral drug treatment in preventing virus escape and persistence. *J. Virol.* 74, 5896–5901
37 Webster, R.G. *et al.* (1986) Vaccination as a strategy to reduce the emergence of amantadine- and rimantadine-resistant strains of A/Chick/Pennsylvania/83 (H5N2) influenza virus. *J. Antimicrob. Chemother.* 18, 157–164
38 von Kleist, M. *et al.* (2011) HIV quasispecies dynamics during pro-active treatment switching: impact on multi-drug resistance and resistance archiving in latent reservoirs. *PLoS ONE* 6, e18204
39 Garbelli, A. *et al.* (2011) Targeting the human DEAD-box polypeptide 3 (DDX3) RNA helicase as a novel strategy to inhibit viral replication. *Curr. Med. Chem.* 18, 3015–3027
40 Geller, R. *et al.* (2007) Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. *Genes Dev.* 21, 195–205
41 Hopkins, S. *et al.* (2010) SCY-635, a novel nonimmunosuppressive analog of cyclosporine that exhibits potent inhibition of hepatitis C virus RNA replication in vitro. *Antimicrob. Agents Chemother.* 54, 660–672
42 Kumar, N. *et al.* (2011) Receptor tyrosine kinase inhibitors block multiple steps of influenza A virus replication. *J. Virol.* 85, 2818–2827
43 Biebricher, C.K. and Eigen, M. (2005) The error threshold. *Virus Res.* 107, 117–127
44 Swetina, J. and Schuster, P. (1982) Self-replication with errors. A model for polynucleotide replication. *Biophys. Chem.* 16, 329–345
45 Holland, J.J. *et al.* (1990) Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis. *J. Virol.* 64, 3960–3962
46 Lee, C.H. *et al.* (1997) Negative effects of chemical mutagenesis on the adaptive behavior of vesicular stomatitis virus. *J. Virol.* 71, 3636–3640
47 Loeb, L.A. and Mullins, J.I. (2000) Lethal mutagenesis of HIV by mutagenic ribonucleoside analogs. *AIDS Res. Hum. Retroviruses* 13, 1–3
48 Crotty, S. *et al.* (2001) RNA virus error catastrophe: direct molecular test by using ribavirin. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6895–6900
49 Severson, W.E. *et al.* (2003) Ribavirin causes error catastrophe during Hantaan virus replication. *J. Virol.* 77, 481–488
50 Sierra, S. *et al.* (2000) Response of foot-and-mouth disease virus to increased mutagenesis. Influence of viral load and fitness in loss of infectivity. *J. Virol.* 74, 8316–8323
51 Sierra, M. *et al.* (2007) Foot-and-mouth disease virus mutant with decreased sensitivity to ribavirin: implications for error catastrophe. *J. Virol.* 81, 2012–2024
52 Airaksinen, A. *et al.* (2003) Curing of foot-and-mouth disease virus from persistently infected cells by ribavirin involves enhanced mutagenesis. *Virology* 311, 339–349
53 Pariente, N. *et al.* (2003) Mutagenesis versus inhibition in the efficiency of extinction of foot-and-mouth disease virus. *J. Virol.* 77, 7131–7138
54 Pariente, N. *et al.* (2001) Efficient virus extinction by combinations of a mutagen and antiviral inhibitors. *J. Virol.* 75, 9723–9730
55 Tapia, N. *et al.* (2005) Combination of a mutagenic agent with a reverse transcriptase inhibitor results in systematic inhibition of HIV-1 infection. *Virology* 338, 1–8
56 Grande-Pérez, A. *et al.* (2005) Suppression of viral infectivity through lethal defection. *Proc. Natl. Acad. Sci. U.S.A.* 102, 4448–4452
57 Grande-Pérez, A. *et al.* (2002) Molecular indetermination in the transition to error catastrophe: systematic elimination of lymphocytic choriomeningitis virus through mutagenesis does not correlate linearly with large increases in mutant spectrum complexity. *Proc. Natl. Acad. Sci. U.S.A.* 99, 12938–12943
58 Loeb, L.A. *et al.* (1999) Lethal mutagenesis of HIV with mutagenic nucleoside analogs. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1492–1497
59 Domingo, E. (2005) Virus entry into error catastrophe as a new antiviral strategy. *Virus Res.* 107, 115–228
60 Anderson, J.P. *et al.* (2004) Viral error catastrophe by mutagenic nucleosides. *Annu. Rev. Microbiol.* 58, 183–205
61 Eigen, M. (2002) Error catastrophe and antiviral strategy. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13374–13376
62 Graci, J.D. and Cameron, C.E. (2002) Quasispecies, error catastrophe, and the antiviral activity of ribavirin. *Virology* 298, 175–180
63 Graci, J.D. and Cameron, C.E. (2008) Therapeutically targeting RNA viruses via lethal mutagenesis. *Future Virol.* 3, 553–566
64 Orgel, L.E. (1963) The maintenance of the accuracy of protein synthesis and its relevance to ageing. *Proc. Natl. Acad. Sci. U.S.A.* 49, 517–521
65 Orgel, L.E. (1973) Ageing of clones of mammalian cells. *Nature* 243, 441–445
66 Manrubia, S.C. *et al.* (2010) Pathways to extinction: beyond the error threshold. *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* 365, 1943–1952
67 Cases-Gonzalez, C. *et al.* (2008) Beneficial effects of population bottlenecks in an RNA virus evolving at increased error rate. *J. Mol. Biol.* 384, 1120–1129
68 Perales, C. *et al.* (2007) Insights into RNA virus mutant spectrum and lethal mutagenesis events: replicative interference and complementation by multiple point mutants. *J. Mol. Biol.* 369, 985–1000
69 Iranzo, J. and Manrubia, S.C. (2009) Stochastic extinction of viral infectivity through the action of defectors. *Europhys. Lett.* 85, 18001
70 Mullins, J.I. *et al.* (2011) Mutation of HIV-1 genomes in a clinical population treated with the mutagenic nucleoside KP1461. *PLoS ONE* 6, e15135
71 Pfeiffer, J.K. and Kirkegaard, K. (2003) A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7289–7294
72 Agudo, R. *et al.* (2010) A multi-step process of viral adaptation to a mutagenic nucleoside analogue by modulation of transition types leads to extinction-escape. *PLoS Pathog.* 6, e1001072

73 Ferrer-Orta, C. *et al.* (2010) Structure of foot-and-mouth disease virus mutant polymerases with reduced sensitivity to ribavirin. *J. Virol.* 84, 6188–6199
74 Perales, C. *et al.* (2009) Potential benefits of sequential inhibitor–mutagen treatments of RNA virus infections. *PLoS Pathog.* 5, e1000658
75 Perales, C. *et al.* (2007) Insights into RNA virus mutant spectrum and lethal mutagenesis events: replicative interference and complementation by multiple point mutants. *J. Mol. Biol.* 369, 985–1000
76 Manrubia, S.C. (2012) Modelling viral evolution and adaptation: challenges and rewards. *Curr. Opin. Virol.* 2, 531–537
77 Ojosnegros, S. *et al.* (2011) Viral genome segmentation can result from a trade-off between genetic content and particle stability. *PLoS Genet.* 7, e1001344
78 Iranzo, J. and Manrubia, S.C. (2012) Evolutionary dynamics of genome segmentation in multipartite viruses. *Proc. R. Soc. Lond. B* 279, 3812–3819
79 Perales, C. *et al.* (2011) Influence of mutagenesis and viral load on the sustained low-level replication of an RNA virus. *J. Mol. Biol.* 407, 60–78
80 Koelle, K. *et al.* (2006) Epochal evolution shapes the phylodynamics of interpandemic influenza A (H3N2) in humans. *Science* 314, 1898–1903