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 mutation rates 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

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\textbf{Glossary}

\textbf{Combination therapy}: treatment that consists of the administration of two or more drugs.
\textbf{Complementation}: increase of viral progeny production mediated by gene products supplied by another virus (in quasispecies, supplied by closely related variants).
\textbf{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.
\textbf{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.
\textbf{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.
\textbf{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.
\textbf{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.
\textbf{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.
\textbf{Lethal mutagenesis}: viral extinction achieved through an excess of mutations, often promoted by mutagenic nucleotide analogs during viral genome replication.
\textbf{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 quasinatural mutations and epistatic interactions in viral genomes, there might be a large ensemble of sequences of almost identical fitness that compose a ‘master phenotype’.
\textbf{Monotherapy}: treatment that consists of the administration of a single drug.
\textbf{Mutant spectrum}: the ensemble of mutant genomes that compose a viral quasispecies. It is also termed mutant swarm or mutant cloud.
\textbf{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.
\textbf{Mutation rate}: the frequency of occurrence of a mutation during viral genome replication.
\textbf{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.
\textbf{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.
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 deflection 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
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 lymphocyctic 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 deflection 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 1). 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 1. 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 (µ) 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:

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

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 1).

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 1. 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 ($t_0$), corresponding to $G$ intracellular replication cycles. Adapted from [29].](image-url)
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
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 non-trivial 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).

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