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Mini-review

Antiviral drug resistance

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

Abstract

Almost 30 years ago it was proposed that the selection for antiviral drug resistance should be used as an indicator of antiviral drug activity. In addition to discriminating between cellular toxicity and specific activity directed against a viral target, drug resistant mutants have been used to confirm the mechanism of action of antiviral drugs, to discover the functions of several viral proteins and to provide insights into viral evolution and fitness. Drug resistance has also become a standard component of both the preclinical and clinical drug development process. For HIV and increasingly for other viruses drug resistance testing has become standard-of-care in clinical practice. A few selected examples are provided to illustrate each of these points.
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In 1977, E.C. Herrmann and J.A. Herrmann published a remarkably insightful essay entitled, "A Working Hypothesis—Virus Resistance Development as an Indicator of Specific Antiviral Activity" (Herrmann and Herrmann, 1977). The article was written largely in response to the extensive literature, which still persists, describing as antivirals compounds that reduce virus replication by host cell toxicity. The Herrmanns proposed that selection for resistant mutants provided proof that the compound inhibited viral functions rather than host cell functions. Audiences at lectures usually consider the statement that "a definition of an antiviral drug is one that selects for drug resistance" to be a joke. However, the Herrmanns' hypothesis about drug resistance has matured into a field that provides major insights into structural biology, virus replication, drug mechanisms, and has become a fundamental component of drug development and patient management.

1. The selection of drug resistant mutants

As reviewed by Herrmann and Herrmann (1977) selection for drug resistant virus mutants in vitro and in vivo had an early history. Mutants of the first approved antiviral drug (amantadine for influenza A) were selected in vitro and were described in 1970 (Oxford et al., 1970). Amantadine and rimantadine were subsequently shown to select for resistance in an animal model (Webster et al., 1985) and in humans (Hall et al., 1987), both with clinical consequences. Unfortunately, human isolates

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of influenza A virus are becoming predominantly adamantane resistant (Bright et al., 2005), especially in China where amantadine is included in over the counter cold remedies, and allegations of its use on chicken farms have been made. Some recent isolates of avian influenza A virus (H5N1) have also been adamantane resistant (Bright et al., 2005). The most extensive systematic analyses of antiviral drug resistance before the antiretroviral era were the studies of the first major success of antiviral drug development, acyclovir. The genetic, pathogenetic and clinical characterization of drug resistance of herpes simplex virus and varicella zoster virus to acyclovir and its two targets, thymidine kinase and DNA polymerase, have provided major insights (Coen, 1996; Gilbert et al., 2002).

In vitro and in vivo selection for drug resistance has become part of the standard of all drug characterization and development as will be summarized below. One critical component of this process is confirmation of the phenotype of a mutation by site directed mutagenesis. Genetic variants are readily generated during the course of viral replication, especially those with RNA genomes (Drake and Holland, 1999). To confirm that a mutation emerged as a consequence of specific selective pressure either in vitro or in vivo as well as what each mutation contributes individually or in combination with other mutations, site directed mutants must be generated to characterize the phenotypic consequences of the mutation before its role as a drug resistance mutation is confirmed.

2. Basic insights into viral protein structure and function and into drug mechanisms of action

There are numerous examples of the analysis of drug resistant mutants providing insights into fundamental aspects of viral protein function. To return to amantadine and influenza A virus, drug resistant mutants were shown to contain point mutations in a recently recognized second reading frame of the M segment of the influenza RNA genome (Hay et al., 1985). This reading frame, designated M2, codes for a tetrameric, transmembrane H^+-ion channel essential for pH mediated changes of the virion that permit entry of the viral ribonucleoprotein into the cytoplasm. Amantadine "plugs" this channel thus blocking virus replication (Holsinger and Lamb, 1991; Duff and Ashley, 1992; Pinto et al., 1992).

Similarly the function of UL97 of CMV was unknown until ganciclovir resistance mutations in that reading frame led to the appreciation that this viral protein was responsible for the anabolic phosphorylation of ganciclovir, and by extension functions as a protein kinase for CMV (Biron et al., 1986).

The first AZT (zidovudine) resistant mutants were isolated from patients participating in the original phase II trial (Larder et al., 1989). The constellation of mutations in reverse transcriptase were quickly identified and associated with phenotypic assays of resistance (Larder and Kemp, 1989). No enzymatic assay, however, could be identified to clearly discriminate wild type and drug resistant reverse transcriptase. The mechanism of AZT-resistance remained elusive for over a decade. In 1998, two sets of investigators explained the mystery (Arion et al., 1998; Meyer et al., 1998). Chain elongation by 3′–5′ phosphodiester bond formation with an incoming dNTP is a reversible process. In the presence of pyrophosphate or ATP, reverse transcriptase can catalytically excise the terminal nucleotide by a process termed pyrophosphorolysis. Given the K_m's of pyrophosphate and ATP for this reaction, and the concentrations of these species in cells, ATP is the likely substrate in vivo. The viral DNA chain is now free to resume elongation with the removal of the chain terminating nucleoside analog. This mechanism of excision repair was appreciated only because of the impetus to explain the mechanism of the drug resistance mutations selected by AZT. These mutations enhance the capacity of reverse transcriptase to perform the excision repair process. Moreover, this mechanism provides an explanation for the cross-resistance of viruses with these mutations to all anti-HIV nucleosides (Whitcomb et al., 2003).

3. Insights into evolution and fitness

The pre-existence of rare, minority resistant variants was first documented in bacteria by Luria and Delbrück (1943) and shown to be clinically important for *M. tuberculosis* in the 1950s (Coates et al., 1953; Cohn et al., 1959). With the high population size of HIV in each patient (Perelson et al., 1996) and the high mutation rate of HIV (Mansky and Temin, 1995), there was every reason to believe that drug resistant mutants pre-exist in the swarm of genetic variants circulating in each patient. Rare drug resistant variants to both nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) have been identified in drug naïve patients (Nájera et al., 1994). A study of the decay of wild type virus and the outgrowth of drug resistant virus with nevirapine monotherapy permitted the calculation of the prevalence of the Y181C mutation in the population of blood plasma HIV RNA in previously untreated patients (Havlir et al., 1996).

Greater rates of virus replication should generate genetic variants more quickly and it was shown early on that patients with more advanced disease developed resistance to AZT monotherapy more quickly (Richman et al., 1990). Stable inducible forms of drug resistant HIV were shown to be established as latent provirus in quiescent CD4 lymphocytes (Wong et al., 1997b; Finzi et al., 1997). We used the M184V mutation that confers lamivudine (3TC) resistance in HIV reverse transcriptase in a variation of a pulse-chase experiment. Studying patients with the M184V mutation who became fully suppressed with combination therapy, we showed that latently infected CD4 lymphocytes are not a homogenous population with a simple exponential decay rate (Strain et al., 2003). Recently established latently infected cells have shorter half-lives than those established at earlier time points. These latter cells may persist for decades to fulfill their role in immunologic memory but unfortunately also maintain latent HIV infection for life. We also showed that suboptimal antiretroviral suppression replenishes the latently infected population (Wong et al., 1997a).

Anatomic compartments play an important role in the dynamics and evolution of HIV. Distinct tissues provide different cell tropisms, immunologic pressures and variations in drug penetration, thus driving evolution differently from virus in the

circulation. We have recently shown that even though each individual's viruses are phylogenetically distinct from those of other individuals, signature polymorphisms can be identified across individuals that distinguish both seminal and CSF virus from virus in the blood (Pillai et al., 2005; Strain et al., 2005). Consistent with earlier studies from others, we found both a high proportion of distinct V3 sequence types in *env* and discordant drug resistance mutations in reverse in *pol* between CSF and blood virus (Wong et al., 1997c; Pillai et al., submitted for publication).

The virus often pays a price to acquire and maintain a drug resistance mutation. The tradeoff between replication fitness and the benefits of drug resistance has been documented by numerous investigators. The M184I mutation emerges most readily to confer rapid resistance to lamivudine during monotherapy of HIV; however, the more fit and equally resistant M184V variant soon supervenes (Schuurman et al., 1995). The number of mutations in protease confers progressively more resistance with progressively less fitness (in the absence of drug). The optimal number of mutations in balancing this tradeoff is determined by the drug concentration. More mutations provide more fitness with increasing drug concentrations (Weber et al., 2003; Gonzalez et al., 2000). The fitness tradeoff of replication capacity versus resistance has been well characterized in patients with multiple drug resistant virus who discontinue their antiretroviral therapy (Deeks et al., 2001).

The role of compensatory mutations in alleviating some of the fitness cost of drug resistance mutations in HIV protease has been well documented. These compensatory mutations can occur in other portions of the protease itself (Nijhuis et al., 1999). Mutations in the protease cleavage sites of *gag* can also mutate to provide better substrates for drug resistant virus, thereby improving replication capacity and fitness (Mammano et al., 1998).

4. Drug resistance in drug development

With the increasing prevalence of both acquired and transmitted drug resistance (discussed below), it has become essential for preclinical drug development to incorporate susceptibility assays into the development process. For decades typical reports of new compounds tested the susceptibility of viruses from different families to characterize the range of activity and specificity of a candidate drug. Now a new candidate HIV drug, for example, will be tested against a series of isolates to show both that natural variation can be addressed within clade B, for example, as well as a panel of wild type isolates representing the global spectrum of clades. Next, the candidate drug will be tested against a series of representative drug resistant isolates. If it is a reverse transcriptase or protease inhibitor, its development is unlikely to be successful if it cannot inhibit most isolates resistant to other drugs in that class. If the candidate is from a new class, documentation of activity against drug resistant isolates is still important.

The design of new protease inhibitors, for example, also includes characterizing the activity against drug resistant enzyme and utilizing crystallography of drug resistant protease. The preclinical development process also now includes the in vitro selection of drug resistant virus to ascertain the speed of development ("genetic barrier") as well as the spectrum of mutations and cross-resistance that emerge with selection.

The clinical development process now requires the testing for resistance in virus that persists with treatment. The mutations that emerge are characterized and the resistant virus must be tested for cross-resistance. Moreover, the strategy of development has been transformed by the prevalence and clinical significance of drug resistance. With the knowledge that monotherapy results in drug resistance, drug development for HIV and HCV restricts the duration of monotherapy to assess the potency of a drug dose to no more than 1 or 2 weeks, after which time the study is terminated or a combination regimen is added to the test drug. Phase III studies of a new antiretroviral drug can pursue two general indications. For "salvage" in patients having failed prior regimens due to drug resistant virus, patients are randomized to an optimized regimen based upon the results of a drug resistance test with or without the addition of the test drug which presumably has activity against drug resistant virus. Demonstration of superior virological endpoints after 24 weeks with continued follow-up for at least a year is considered supportive of an unmet medical need to fulfill criteria for registrational approval. The second major indication is of course initial therapy which now requires a drug resistance test as discussed below.

5. Drug resistance in clinical management

HIV drug resistance is both a major consequence and cause of HIV treatment failure (Hirsch et al., 1998, 2000, 2003). The prevalence of acquired drug resistance (although emerging less frequently with the availability of better regimens and with patients not previously exposed to nucleosides only) is remarkably high in patients not fully suppressed on potent combination therapy (Richman et al., 2004). The prevalence of transmitted drug resistance increased throughout the 1990s, but appears to have plateaued with variable frequencies, usually 10–20%, throughout Europe and North America (Pillay and Zambon, 1998; Little et al., 1999, 2002). Some of the factors that contribute to this plateau could be the reduced fitness of some drug resistant viruses and the increasing proportion of patients who initiate antiretroviral therapy and suppress virus replication, thus not becoming potential transmitters of drug resistant virus (Leigh Brown et al., 2003). Although most drug resistance has been described in clade B infections in North America and Europe, clade C HIV is responsible for over half of the infections word-wide (Osmanov et al., 2002). Most areas where non-clade B infection is endemic have remained without access to antiretrovirals, but this is changing, and it is expected that HIV drug resistance will become an increasing problem there as well (Kantor and Katzenstein, 2004).

As a result, the paradigm of combination chemotherapy to prevent the emergence of resistance, proven decades ago for the management of tuberculosis and tumors, has become the standard of practice for HIV chemotherapy (Gulick et al., 1997; Hammer et al., 1997). The use of drug resistance assays to select regimens to contend with both acquired and transmitted drug

resistance has become the standard-of-practice (Hirsch et al., 1998, 2000, 2003). Of note, the strategy to address drug resistance by interrupting treatment to permit the reemergence of wild type virus has proven ineffective at best and potentially dangerous for the patient (Lawrence et al., 2003).

Drug resistance is becoming increasingly well recognized and characterized for the expanding numbers of antiviral drugs. The practical information for clinical management involves both epidemiology and individual clinical management. This information applies not only to HIV, as discussed above, but to influenza virus (Bright et al., 2005; Beigel et al., 2005; Hayden et al., 2005), the herpesviruses including HSV 1, HSV 2, VZV and CMV (Gilbert et al., 2002), hepatitis B virus (Locarnini et al., 2004) and in the near future hepatitis C virus.

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