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A Few Specialized Issues That Should Be Focused on Anti-HIV Drug Evaluation In Vitro*

Dao-min ZHUANG and Jing-yun LI **

(State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Academy of Military Medical Science, Beijing 100071, China)

Abstract:Since the introduction of antiretroviral therapy (ART), the lifespan and quality of life of patients infected with HIV have been significantly improved. But treatment efficacy was compromised eventually by the development of resistance to antiretroviral drugs, and more new anti-HIV drugs with lower toxicity and higher activity were needed. Based on the experience and lessons learned from the treatment in the developed countries, US FDA suggested that more pharmacodynamical researches should be considered ahead of the clinical trials. To facilitate the anti-HIV drug research and development, we reviewed a few specialized issues that should be focused on drug evaluations *in vitro*, including: 1) Mechanism of action studies, demonstrating the candidate drug's efficacy to specifically inhibit viral replication or a virus-specific function and confirm the drug target. 2) Drug resistance studies, selecting the drug-resistant variants in vitro and determining the activities inhibiting HIV isolates resistant to approved antiretroviral drugs of the same class. 3) Antiviral activity in vitro in the presence of serum proteins, ascertaining whether an investigational product is significantly bound by serum proteins. 4) Combination activity analysis, evaluating *in vitro* antiviral activity of an investigational product in two-drug combinations with other drugs approved.

Key words: Human immunodeficiency virus; Drug evaluation; Drug resistance

There are no ideal animal models for HIV infection. Pharmacodynamics (PD) research in vitro has a critical role in evaluating antiviral activity and being permitted to clinical trials, besides the general antiviral activity evaluation in cell culture level, more attentions should be focused on a few specialized issues as following:

MECHANISM OF ACTION STUDIES

Because of the high mutation rate of HIV, combinations of antiretroviral drugs that target different stages of virus lifecycle are now used for the treatment of HIV infection — so-called highly active antiretroviral therapy (HAART). The use of agents from different classes is instrumental in controlling

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** Corresponding Author.
Phone: +86-10-66948566, Fax: +86-10-63842689,
E-mail: lijy@nic.bmi.ac.cn

virus replication and suppressing the infection. Therefore, researches on the drug target can help us to determine which drugs should be combined, to predict drug toxicities and the genetic domains associated with drug resistance. To elucidate the mechanisms of action, or the site of the drug's action at least, is an essential content of PD [1, 2].

Mechanism of action studies include that demonstrating the candidate drug's efficacy to specifically inhibit viral replication or a virus-specific function and confirm the drug target, for example, viral reverse transcriptase or protease. Methods that demonstrate the mechanism of action include receptor binding, inhibition of enzymatic activity, X-ray crystallographic structure determination of bound inhibitor complex, and characterization of resistance mutations in the gene encoding the target. A clear understanding of the mechanism of action of antiviral drugs can provide insight into the regions of the viral genome where resistance mutations could develop. These regions are not limited to viral-encoded target of the candidate drug but can include the enzyme substrate or another viral-encoded protein existing in a complex with the target protein. The specificity of the candidate drug should be demonstrated for the viral target over host cellular proteins, especially in those cases in which a viral enzyme has a cellular counterpart. For example, if a candidate drug targets a viral polymerase, the activity of the drug against the viral polymerase should be determined and be compared with its activity against host DNA polymerases.

DRUG RESISTANCE STUDIES

Because of the high genetic mutation rate and drug pressure, HIV develops resistance mutations, even cross resistance mutations, soon after the antiretroviral therapy (ART). Cross-resistance, defined as resistance to drugs to which a virus has never been exposed, results from mutations that have been selected for by the use of another drug. The emergence of drug resistance is inevitable, but can be delayed by the combination chemotherapy. With the natural variations and transmission of drug resistance strains, many treatment-naïve patients infected with resistant strains. In the developed countries where ART is widely applied, HIV strains with resistance to single or multiple antiretroviral drugs are more and more evident in the newly diagnosed persons. The roll-out of free ART of China began in 2002 and the prevalence of resistant strains has reached 50-60% in the central areas, though which is relatively low in the whole country. Consequently, the prevalence of drug-resistant strains needs more concerns for the new drug research and PD studies.

The significance of drug resistance studies in antiviral drug research: (1) elucidate to which extent the virus develops resistance to a drug, the patterns and types of mutations, and to direct judging the genetic barrier to resistance and designing the genotyping method. (2) to elucidate the cross resistance to other drugs in the same class, to guide the determination the clinical symptoms corresponding to this drug, to guide the selection of drug combination, what kind of drug could be combined to avoid the drug resistance and to achieve the maximum efficacy. For example, to evaluate the efficacy of a new drug in phase 1 and 2 trials, we should enroll subjects with confirmed genotypic and/or phenotypic resistance of the same class. But in phase 3, enrollment should be

restricted to subjects without these mutations at baseline. FDA suggested that drug resistance research should be conducted throughout the whole course of drug research and development. At the early stage of drug research and development, the potential of drug resistance strains selection and the inhibition efficacy to drug resistance strains should be evaluated[8, 9].

Selection of resistant virus *in vitro*

Selection of resistant virus in cell culture system could determine the genetic barrier for resistance development is high or low. A candidate drug with a low genetic threshold may select for resistance with only one or two mutations, while a product with a high genetic threshold may require multiple mutations to facilitate resistance. There are two basic methods to isolate drug resistant variants. One is that using a high initial virus inoculum and being propagated for several passages at a fixed drug concentration, using multiple cultures to test different concentrations. It is suitable for the candidate drugs with a low genetic threshold. The other is that using a low initial virus inoculum and being passaged in the presence of increasing product concentrations starting near the double EC_{50} values for the parental virus. The drug resistance is identified by genotypic and phenotypic assay. The emergence of resistance mutations is a proof of antiviral activities, which could validate the drug's specific viral target.

In genotyping assay, the complete coding sequence of the gene encoding the target protein is obtained and the mutations associated with reduced drug susceptibility are characterized. Then the mutations are introduced into a recombinant virus system by using site-directed mutagenesis and polymerase chain reaction (PCR) amplification for measuring susceptibility to the candidate drug in vitro, and then the fold-changes in IC_{50} value for recombinant virus relative to the wild-type are calculated. At present, a new class of compounds that block the binding of virus to the CCR5 cellular co-receptor has been approved, which is the first example of a drug that blocks a cellular protein rather than acting directly on a viral component. The co-receptor change may indicate the resistance to this class of drugs. For CCR5 antagonist, maraviroc, there are two mechanisms by which resistance emerges. First, viruses that switch to CXCR4 or are dual tropic emerge during therapy. The second mechanism is via alterations in the amino acid sequence of the V3 loop which then allow the virus to bind to the CCR5 with the inhibitor bound in place. So, the tropism and co-receptor change should be examined in drug selection experiments of this class of drugs.

Drug resistance and cross-resistance evaluation

Antiviral products targeting the same protein (typically products of the same drug class) may develop mutations that lead to reduced susceptibility to one antiviral product and can result in decreased susceptibility to other antiviral products in the same drug class. When new variants resistant to a candidate drug are defined, the susceptibility to other drugs in the same class should be determined. Vice versa, the susceptibility of approved drugs-resistant viruses to a candidate drug should be determined.

SELECTION OF HIV ISOLATES OF PD IN VITRO

There are many genotypes of HIV and more circulating recombinant forms (CRFs) are emerging. ART mainly was applied in developed countries, where HIV-1 subtype B was dominant. Non-B

subtypes are dominant in Africa and Asia, more information is needed on the natural susceptibility of non-B subtypes and on the patterns of resistance mutations that occur in these strains, which can differ from those observed with subtype B. theoretically, the target genes of ART vary like other viral genes, which maybe influence susceptibilities to candidate drugs. Some strains of HIV-1 group O are naturally resistant to some NNRTIs. HIV type 2 (HIV-2) is intrinsically resistant to most nonnucleoside reverse transcriptase inhibitors and a protease inhibitor, amprenavir. Similarly, some strains of HIV-1, CRF01-AE and CRF02-AG can be less susceptible to abacvir, atazanavir than the subtype B strains. The susceptibilities of different subtypes of strains to antiviral drugs need to be well characterized [7, 12, 13, 18].

For the diversity of HIV and popularity of resistant viruses, susceptibility of different strains may vary and resistant strains widely persist. It is recommended by FDA that 50-100 laboratory strains and well-characterized clinical isolates, including T-tropic, M-tropic, or B and non-B subtype, HIV-2 [1, 2]. Resistance strains and clinical resistant isolates are also needed, as well popular HIV strains in the area of clinical trials. For maraviroc, a CCR5 antagonist of Pfizer, 50 clinical isolates and 200 recombinant viruses were used in antiviral activity research. 50 clinical isolates included: 44 of R5-tropic, 4 X4-tropic and 2 dual-tropic isolates. OR 25 of subtype B, 4 group O, 4 subtype C, 2 subtype F, 6 subtype D,2 subtype G, 4 subtype A, 2 subtype J and 1 CRF01-AE by sorts of subtypes. 200 recombinant viruses is constructed with different *env* genes, included: 160 subtype B and 40 non-B subtype, or 100 wild- and 100 drug resistant-strains [17]

PD OF CANDIDATE DRUGS WITH DIFFERENT MECHANISMS

A candidate drug should be established antiviral activity in vitro irrespective of its mechanism of action, genotypes and phenotypes of viral strains. For drugs with different mechanisms, we should choose resistance strains with same target proteins, e.g. a reverse transcriptase inhibitor, we should select strains resistant to approved nucleoside reverse transcriptase inhibitors or nonnucleoside reverse transcriptase inhibitors. In addition, we should conform that the candidate drug specifically acts on the target, e.g. a protease inhibitor should specifically inhibit viral proteolytic activity.

ANTIVIRAL ACTIVITIES OF DRUGS IN THE PRESENCE OF SERUM PROTEINS

The binding of drugs to plasma proteins need to be considered during drug research and development [1]. Every drug will bind to the plasma proteins more or less, and only unbound drugs can penetrate the target cells and exert the therapeutic effect. Plasma proteins can bind many antiviral drugs and interfere with the antiviral activities, so that we must improve the doses to counteract the effect. For most research, ascertain whether a candidate drug is significantly bound to serum proteins. Common methods for determining protein binding include equilibrium dialysis, ultrafiltration methods, and fluorescence-based high throughput albumin and α1-acid glycoprotein protein binding. Studies of plasma protein binding mainly evaluate binding to α1-acid glycoprotein (AAG) and human serum albumin (HSA) [2, 3]. AAG, which accounts for only about 1%-3% of plasma proteins, binds drug molecules with low capacity but high affinity, with the

latter characteristic making dissociation of the drug molecule from AAG more difficult than from albumin. Although albumin is a major protein component of plasma, it is a high- capacity but low-affinity binder. If the investigational product is highly protein bound, we should examine the *in vitro* antiviral activity of the investigational product in the presence of a series of dilutions of human serum up to 40-50 percent (e.g., 5 %, 10 %, 20 %, and 40 %). An EC_{50} value for 100 % human serum can be extrapolated from these data and the serum-adjusted EC_{50} values are reported. In addition, sponsors are encouraged to determine EC_{50} values in the presence of physiological concentrations of AAG and HSA.

EFFICIENCY EVALUATION OF DRUG COMBINATION

A combination of two or more drugs is widely applied to clinical therapy, especially in the treatment of cancer, use of antibiotics and antiviral therapy. The routine anti-HIV therapy is a combination of three drugs targeting different stages of virus replication. It is recommended by many famous institutions and international organizations that a new candidate drug should be tested its antiviral activity in two-drug combinations with approved antiretroviral drugs for the interactions of products are complex and can result in antagonistic, additive, or synergistic effects with respect to antiviral activity [9, 15]. For this reason, sponsors should evaluate the in vitro antiviral activity of investigational products in two-drug combinations with other products approved for the same indication. Specifically, combinations that should be tested include the investigational product with all approved products that target the same protein and at least two appropriate products from each class of products approved for the same indication. We recommend completing the in vitro drug combination activity studies of the candidate drug with approved products before initiation of clinical trials that will evaluate the efficacy of the investigational product in combination with other antiviral products [8].

There are two types of models to evaluate drug combination effect, Bliss independence and Loewe additivity[4, 19, 20]. A variety of particular methods can be traced back to these two basic approaches. Bliss independence is widely used in radiation research, while the latter is the gold standard in other fields. Many methods to analyzing drug combination have relations with Loewe additivity model, such as the median-equation approach developed by Chou and Talary, where combination index (CI)<1, =1, and>1 indicate synergism, additive effect, and antagonism, respectively[6, 17]. Another method is isobologram, which is the prototype of MacSynergy Ⅱ software.

The three-dimensional (3-D) methods have more advantageous comparing to traditional two-dimensional (2-D) methods, since the combined-action experiments involves three variables, the respective drug concentrations and the biological effect. First, the dose-response surfaces obtained by 3-D methods visualize the complex drug-drug interactions accurately. Secondly, the shapes of dose-response surfaces reflect the combination effect. Thirdly, the results can be quantified, so which could be compared with each other. Finally, the 3-D methods contain more information for various combinations of two drugs' concentrations. Moreover, the results could verify which combination is statistically significant, while other methods could not.

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