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Selection and analysis of HIV-1 integrase strand transfer inhibitor resistant mutant viruses

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ABSTRACT

This report describes methods for the selection and analysis of antiretroviral resistance to HIV integrase strand transfer inhibitors (InSTIs) in cell culture. The method involves the serial passage of HIV-1 in the presence of increasing concentrations of test inhibitors, followed by the cloning and sequencing of the integrase coding region from the selected viruses. The identified mutations are subsequently re-engineered into a reference wild-type molecular clone, and the resulting replication capacity and level of drug resistance are determined relative to the wild-type virus. Here we describe examples of selection and analysis of InSTI-resistant viruses using four integrase inhibitors from three structurally distinct chemical classes; a diketo acid, two naphthyridines, and a pyrimidinecarboxamide. Each inhibitor selected an independent route to resistance. Interestingly, the shift in the IC_{50} required to suppress the re-engineered resistant mutant viruses closely matched the concentration of compound used during the selection of drug resistance.

1. Introduction

The efficacy of antiretroviral agents can be compromised by the emergence of HIV variants that are resistant to the agent. In some cases, these variants may also be cross-resistant to other agents in the same class, seriously limiting treatment options for patients infected with multi-drug-resistant viruses. Approaches to address this problem include the development of new compound classes and the development of next-generation inhibitors in existing classes that retain substantial activity against known resistant viruses. In either case, it is critical to understand how antiviral resistance may emerge to those new agents, information that can be obtained by intentionally selecting for resistant viruses in cell culture. The selection of resistant viruses in cell culture can also provide important information about a compound's antiviral mechanism of action.

Resistance arises through a process of variation and selection: the relatively poor fidelity of the HIV-1 reverse transcriptase (RT) leads to frequent mutations in the viral genome, at a rate of about 0.85 bp/genome/replication cycle [1]. Following multiple replication cycles, a quasispecies of viruses containing a large number of genetic variants arises. While many of these mutations may be lethal or deleterious to viral replication, some may confer resistance to antiviral agents. Mutations in viral proteins can confer drug resistance by various means including interfering with drug binding to the viral protein. However, the same mutation that confers resistance may also result in reduced function of the viral protein and, consequently, the ability of the virus to replicate. Understanding the effects of resistance mutations therefore requires measurement of (minimally) two different properties of the virus, the degree of drug resistance and the ability of the virus to replicate in cell culture.

Resistance is often expressed as the fold-change (FC) in 50% inhibitory concentration (IC_{50}) ($FCIC_{50}$), defined as the IC_{50} of an antiviral agent against a mutant virus divided by the IC_{50} of the agent against a reference wild-type virus. The ability of the virus to infect cells is often expressed as replication capacity (RC), which is measured in different ways depending on the HIV infection assay used (see below for more detail).

There are generally two methods to select for antiviral resistance, which we will refer to as a dose escalation method and a high multiplicity-of-infection (MOI) method. For dose escalation, infection is carried out typically at low MOI (0.01–0.5 infectious units/cell) and at low concentration of inhibitor (at most five times the IC_{50}). Since the virus is allowed to replicate at low-levels, any mutation conferring a slight increase in resistance or RC confers a replication advantage and is therefore actively selected. The cells or virus-containing supernatants are then passaged at regular intervals until the entire culture is infected. Once virus breakthrough is complete, the supernatant is passaged onto fresh cells, additional inhibitor is added (typically at some multiple over the initial concentration), and the culture is passaged again until virus breakthrough reoccurs. As this procedure is repeated, the virus

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population becomes increasingly resistant to the test inhibitor. At various times in this procedure, the virus supernatant is analyzed for the appearance of mutations in the therapeutic target. Once mutations have become established and identified, they are re-engineered into a wild-type proviral clone. Viral stocks are generated and tested for RC and $FCIC_{50}$ values relative to the wild-type.

High MOI experiments are typically performed at a single concentration of compound (e.g., at 100-fold above the IC_{50}) [2]. The input virus is inoculated at 0.1–5 infectious units/cell. Infecting cultures with a large virus inoculum increases the probability that a drug-resistant mutant will be present in the initial infection, and any pre-existing resistance mutants will be selected in a relatively short period of time. This approach is most useful when only one or two mutations are required for resistance. If multiple mutations are required for high-level resistance, the probability of those mutations pre-existing in the virus stock is low.

Integrase activity is required for the productive replication of all retroviruses, including HIV [3]. The enzyme, which is present in virus particles, has two distinct catalytic activities: 3′ processing (removal of the terminal two deoxyribonucleotides from each 3′ end of the linear viral DNA) and strand transfer (covalent joining of the recessed 3′-hydroxyl groups to the 5′-phosphates of a double stranded staggered cut in chromosomal DNA – refer to other papers in this issue of *Methods* for mechanistic details on integrase function).

Clinically relevant integrase inhibitors preferentially target the DNA strand transfer step of integration, and are collectively referred to as integrase strand transfer inhibitors (InSTIs). The first class of sub-micromolar InSTIs was the diketoacid (DKA) derivatives such as compound **1** [4,5] (Fig. 1). Another more potent class of inhibitors are the naphthyridine carboxamides (Fig. 1, compounds **3** and **4**), exemplified by L-870,810 (compound **3**), the first InSTI to show clinical efficacy [6,7]. The first InSTI with clinical approval for treating HIV-1 infection was raltegravir [8], a pyrimidinecarboxamide structurally related to compound **2**.

Here we describe the dose escalation method for resistance selection, as well as downstream analyses of how these changes effect HIV-1 sensitivity to InSTIs. Each inhibitor class selected a distinct pathway to resistance. A preliminary characterization of the profiles of viruses selected in the face of inhibitors **1** and **3** was described previously [7]. Here we provide additional details of that analysis and include data for two additional compounds, a naphthyridinecarboxamide (Fig. 1, compound **4**) and pyrimidinecarboxamide (compound **2**), the latter of which is structurally related to the recently approved drug raltegravir.

2. Methods

2.1. Compounds

Compounds (Fig. 1) were synthesized by the Medicinal Chemistry Department at Merck Research Laboratories, West Point, PA and Rome, Italy.

2.2. Cell culture

H9 T cells (ATCC, Manassas, VA) were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. P4/R5-derived HeLa cells (a generous gift from N. Landau) were cultured in DME media (Mediatech, Manassas, VA) supplemented with 10% FetalClone I (Thermo Scientific Hyclone, Logan, UT), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml puromycin. 293T cells were maintained in DMEM (Lonza, Basel, Switzerland) supplemented with 10% FBS. All cells were maintained at 37 °C in a humidified, 5% CO_2 incubator.

2.3. Virus

H9 cells infected with HTLV-IIIB (ATCC) were propagated in H9 cell media (above). NL4-3 derivatives of HIV-1 were produced by transient transfection (see below).

2.4. Resistance selection

Selection of drug resistance was conducted in H9 cells by mixing 6×10^5 cells with 6×10^3 H9/IIIB infected cells (1:100 ratio), and centrifuging the cells in a microcentrifuge tube at 5000–6000 rpm for 5 min. The cell mixture was washed once with phosphate-buffered saline (PBS), resuspended in complete RPMI media containing either no drug or the starting amount of inhibitor, and 2.4×10^5 cells were transferred to a 48 well plate in 1 ml of media.

Infections were maintained by removing half of the cells and refeeding the remaining cells every 3–4 days with an equal amount

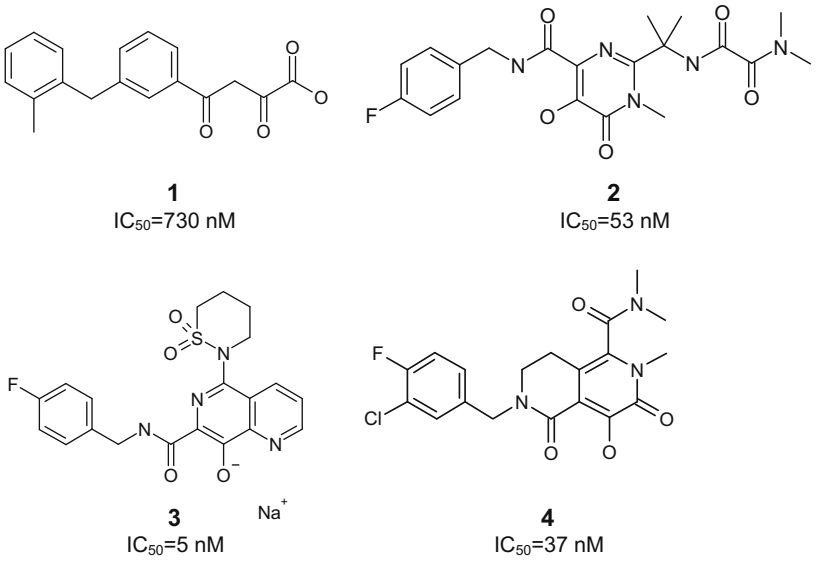

Fig. 1. Structures and antiviral activities of the compounds used in this article: **1**, diketoacid (DKA) [5], **2**, pyrimidinecarboxamide [8], **3** and **4**, naphtyridinecarboxamides [7,11], including L-870,810 (compound **3**).