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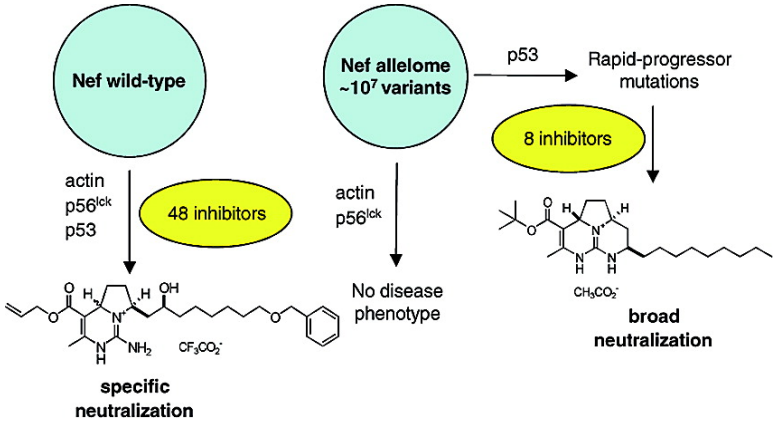

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Library versus Library Recognition and Inhibition of the HIV-1 Nef Allelome

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Current antiviral therapies become ineffective largely due to the accumulation of mutations conferring drug resistance.[1] This enormous problem also confronts other treatments that allow selection for drug-resistant phenotypes. For example, resistance to antibiotic and anticancer compounds can result from evasion of the treatment by surviving cells.[2,3] To slow the development of drug resistance, anti-HIV treatments often apply cocktails of multiple drugs (HAART).[4] Expanding the anti-HIV repertoire to include inhibitors of other HIV proteins, such as Nef, could expand treatment options. New inhibitors would also be susceptible to the evolution of drug resistance. Targeting HIV Nef with small molecules screened against a combinatorial library of Nef variants found in the clinic could provide a more effective strategy for identifying antiviral treatments less vulnerable to the development of drug resistance (Figure 1).

Although many cellular binding partners to Nef have been identified,[5−7] the relative importance and contributions of Nef−ligand interactions to the HIV lifecycle remain incompletely understood. Nef interactions with CD4[8] and major histocompatibility complex class I molecules (MHC-I)[9] can lead to their down-regulation. In some assays, Nef binding to p53 can block p53-mediated apoptosis.[10,11] The interaction with actin could also influence subcellular localization of Nef,[12] and the interaction with $p56^{lck}$ leads to endocytosis of CD4.[8] Nef expression, however, is clearly essential for propagation and maintenance of viral loads.[13] Variations in Nef sequences isolated from HIV-infected individuals can also correlate with different rates of HIV progression.[14]

Inhibiting Nef could potentially replicate the effect of harboring a *nef* deletion or a nonprogressor Nef sequence, both of which are known to hinder progression to AIDS.[14] Recently, we demonstrated guanidine alkaloid-mediated inhibition of full-length HIV-1 Nef (HIV_{NL4-3} isolate, referred to here as wild-type or wt Nef) binding to p53, actin, and $p56^{lck}$.[15] Although the cytotoxicity of batzelladine and crambescidin Nef inhibitors prohibit in vivo studies, the compounds can be used for in vitro drug resistance studies. We asked whether a library versus library approach could simultaneously uncover structure−activity relationship information about the Nef inhibitors. Library inhibition could also suggest a broadly neutralizing inhibitor that is potentially less susceptible to the development of resistance. This approach is inspired by the immune system, which deploys a library of antibodies in response to a viral attack. Broad antiviral neutralization can result from an antibody targeting the viral protein residues required for function.[16] Before seeking a small-molecule analogue to broadly neutralizing antibodies, a library of Nef variants was constructed for functional selections.

We generated a pool of phage-displayed Nef variants from clinical isolates. In an approach complementary to previous drug resistance studies with random mutagenesis,[17] we focus on known Nef mutations. Termed an "allelome," this library includes mutational variants, allotypes, of a single protein. Nef allelome design

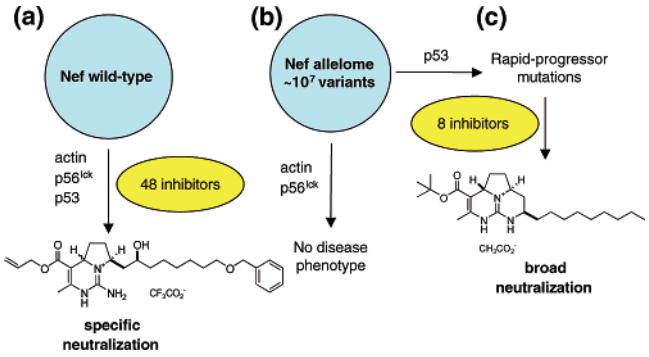

Figure 1. Broad-spectrum allelome inhibitor discovery. (a) Inhibition of wt Nef scored compound effectiveness. (b) Nef allelome selections from binding to p53 harbored Nef mutations associated with rapid progression to AIDS. (c) Next, competition assays examined inhibition of the Nef allelome.

and construction focused on the N-terminus of Nef, an essential region for binding to p53, actin, and $p56^{lck}$,[10,12,18] (Supporting Information). Nef sequences differ among HIV patients with non- or rapid-progression to AIDS,[14] although the mechanistic basis for the variation remains unknown. In addition to the Nef mutations associated with AIDS progression, the library included other mutations observed clinically. The resultant Nef allelome required $\sim 10^7$ different combinatorial variants of Nef. This diversity was accessed by oligonucleotide-directed mutagenesis[19] and the resulting phage-displayed protein libraries.

Next, functional Nef variants were selected by panning the allelome against p53, actin, or $p56^{lck}$. This selection can identify invariant, critical residues for each interaction and can also potentially correlate disease-associated mutations with binding to a particular ligand. Four or five rounds of selection identified functional Nef variants capable of binding to p53, actin, or $p56^{lck}$. The Nef N-terminus is required for high-affinity binding to the target ligands, and also has three residues implicated in the rapid progression to AIDS. We were interested in whether the functional Nef variants from the selections would also correlate with known progressor sequences.

Initial selections against p53 showed strong preference for Nef allelome sequences associated with rapid progression to AIDS (residues A15, R39, and T51; Supporting Information). Selections for Nef allelome binding to actin showed no clear preference for mutations associated with either rapid or nonprogression to AIDS. As an intermediate case, Nef interactions with $p56^{lck}$ selected mutations associated with rapid progression (15 and 39) and one residue (N51) associated with nonprogression. Subsequent selections for Nef allelome members binding to actin and $p56^{lck}$ correlated with initial trends, and two additional independent selections for binding to p53 revealed that residue 51 did not have a strong preference for the threonine mutation associated with progression. The selection for rapid progressor sequences suggests that the Nef-

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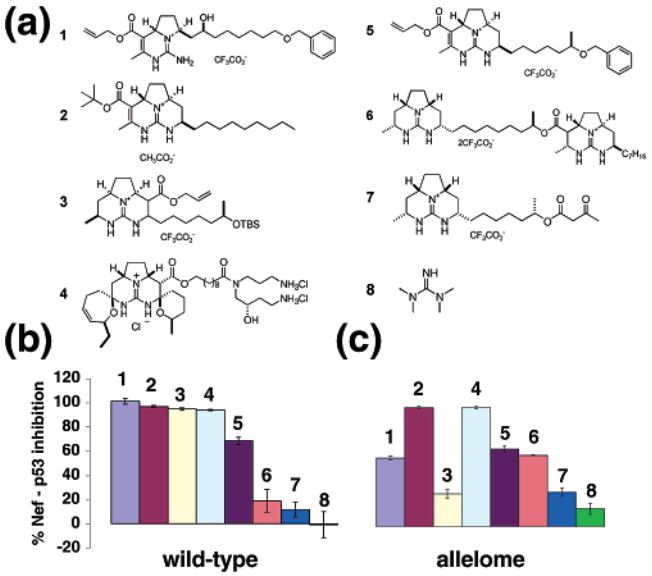

Figure 2. Inhibition of the Nef allelome with 5 μM compound. (a) Compounds assayed for inhibition. (b) Inhibition of wt Nef and (c) Nef allelome binding to p53. Error bars show standard error.

p53 interaction is important for rapid progression of HIV, with residues 15 and 39 potentially critical for the protein−protein interaction. The result suggests future studies focus on the Nef−p53 interaction to determine Nef contributions to the rapid progression to AIDS.

Small-molecule competition ELISAs[15] examined inhibition of the Nef allelome, following selection for binding to p53. To manage the large numbers of potential receptor−ligand combinations, inhibition data against wt Nef allowed division of the small-molecule library into groups of effective (**1**−**4** with >90% efficacy at compound concentrations of 5 μM against wt Nef), moderate (**5**), and poor/negative control inhibitors (**6**−**8**)[15] (Figure 2). Across different selections, the percent inhibition for the Nef allelome was remarkably consistent; for example, alkaloids 1 and 2 inhibited two different selections of the Nef allelome with inhibition ranging from 44.9 to 53.8% and 90.4 to 93.3%, respectively.

Alkaloids **2**, **4**, and **5** were equally effective against both wt and allelomic Nef. The control inhibitors, **6** and **7**, had inconsequential activity (Figure 2). Alkaloids **1**, **2**, and **3** share similar structural properties and likely bind to the same region of Nef. However, alkaloid **2**, the only one of the three effective against the allelome, is structurally the simplest and most flexible compound, lacking phenyl or tributyl silyl ether functionalities. We hypothesize that alkaloid **2** can more precisely target the Nef residues required for functional p53 binding. Alkaloids **1** and **3** can still interact with the Nef allelome, but are more easily dislodged by mutations surrounding key residues required for Nef binding to p53. Control experiments demonstrate the compounds fail to inhibit the Vif−p55 gag interaction, and the addition of Triton X-100[20] has no effect on inhibitor activity (Figure S1, Supporting Information).

As demonstrated here, a compound with fewer functionalities can offer the broadest spectrum of inhibition against the Nef allelome. Studies of HIV-1 protease inhibitors and drug-resistant protease variants have also suggested the importance of well-placed, flexible functional groups in adapting to resistance-conferring mutations.[21] However, suggesting the need for further studies, the complex alkaloid **4** is effective against the both wt and allelomic Nef. Such broad-spectrum inhibitors need to target the few functional groups required for protein function. By targeting a limited number of highly conserved functional groups, the inhibitors can more effectively block the Nef allelome. When applied to a library of proteins with an assortment of substitutions at nearby positions, inhibitors that cannot recognize and adapt to a new binding surface, such as alkaloids **1** and **3**, will lose effectiveness. The libraries versus libraries approach demonstrated here provides further evidence for the abilities of adaptive inhibitors and demonstrates that simple, flexible compounds can be the most effective inhibitors against a library of protein variants.

In summary, we demonstrate a new method for developing broad-spectrum antiviral compounds. This method could also find broad applicability for discovery of more effective antibiotic and anti-cancer compounds. Furthermore, given the expedience of the method (less than one month from library construction to inhibition assays under ideal conditions), library versus library techniques could be used in the early stages of pharmaceutical development. By isolating phage-displayed variants remaining bound to the target ligand in the presence of the inhibitor, drug-resistant protein variants could also be readily identified for further compound optimization and study.

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Supporting Information Available: Additional control experiments, representative experimental procedures, and sequences of Nef selectants. This material is available free of charge via the Internet at http://pubs.acs.org.

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