with a novel conformational structure formed upon coculture of virus and cells. The pyrimidinediones inhibit the replication and transmission of resistant viruses bearing RT or Env mutations alone (albeit with 100-fold loss of sensitivity to NNRTI-resistant viruses) and remain highly active against multi-drug resistant (MDR) viruses with mutations in RT and/or protease. With serial passage in increasing compound concentrations, a virus which is completely resistant to the selecting pyrimidinedione can be selected. The selection follows a defined progression consisting of the initial appearance of mutations in the RT, resulting in approximately 100-fold loss in sensitivity, followed by the accumulation of mutations in gp120, gp41 and gag proteins which allow the virus to escape entry or maturation inhibition, yielding viruses with 1000–10,000-fold resistance. Finally, multiple additional changes occur in RT, resulting in complete resistance. The mutational profile suggests that the compounds act as typical NNRTIs but target a unique conformational structure to inhibit entry requiring interaction of envelope and gag proteins. Each of the resistant viruses have been evaluated for their sensitivity to other nonnucleoside, nucleoside and nucleotide RT inhibitors, entry and fusion inhibitors, and protease inhibitors. Cross-resistance is only detected with other NNRTIs. In light of the ability of the pyrimidinediones to inhibit entry and cell–cell fusion, it is notable that the viruses do not have cross-resistance to Fuzeon. The pyrimidinediones thus represent excellent therapeutic and microbicide development candidates based on the inability of viruses resistant to one of the mechanisms of action to abrogate the activity of the second mechanism. The pyrimidinediones have the potential to replace Sustiva and Fuzeon in current therapy regimens, but with one small molecule with a higher genetic barrier to resistance.

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Azaindole-based HIV-1 Integrase Specific Inhibitors Display Potent Anti-Retroviral Activity

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We have synthesized and studied a series of compounds that share an azaindole core and additional groups such that the expected conformation would form a two-metal binding motif. We the characterization of the representative compound, PF-00558475, demonstrating that it is a potent and selective inhibitor of the strand transfer activity of HIV-1 Integrase and displays corresponding viral activity in cell culture HIV infections. We present biochemical, antiviral, and mechanistic studies indicating specific inhibitory activity against the HIV-1 Integrase enzyme. In addition, we have carried out resistance selection studies confirming that mutations to the Integrase gene are necessary and sufficient to reduce HIV-1 susceptibility to PF-00558475. Finally, we present results from a panel of clinical isolates indicating that this compound is a broad spectrum inhibitor of HIV-1.

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Establishment of a Cell-based HTS System for Discovery of Anti-Flavivirus Drugs

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Flavivirus infections have become a global public health concern due to the severe nature of disease caused by these viruses and their prevalence in the human population. There is an urgent need for specific antiviral therapies to treat these infections. The viral NS3 serine protease is an attractive target for anti-flavivirus therapy because it is highly conserved and essential for viral replication. We have designed a cell-based high throughput screening system for the identification of small molecule inhibitors of the flavivirus protease. In this system, two expression constructs were generated that constitutively express a bi-cistronic mRNA encoding the viral protease; or a proteolytically inactive mutant protease, and a marker gene (GFP^{CSI}) containing a specific cleavage-site for the viral protease inserted within the GFP. Cleavage at this site by the viral protease results in a loss of GFP fluorescence. Coexpression of NS3 protease of Dengue Virus type 2 with the engineered GFP^{CSI} in 293T cells results in site-specific cleavage of GFPCSI, destabilization of GFPCSI conformation, and reduced fluorescence. The relative fluorescent signal for GFPCSI in cells expressing wild-type NS3 (2.3 MFI) was reduced to $72 \pm 5\%$ of that in cells expressing the active site mutant $NS3^{S135A}$ (3.2 MFI) as measured by flow cytometry. The system has been validated by quantifying the level of inhibition of proteolytic activity in the presence of known protease inhibitors and experimental compounds. In the presence of Aprotinin, a known serine protease inhibitor, the proteolytic inhibition was 67%. Likewise, an inhibitor compound (ST905) identified by high throughput screening that reduced NS3-specific protease activity by 51% at 5 μM showed an 18% inhibition of GFP cleavage in the cell-based assay. Taken together, the cell-based HTS system will facilitate discovery of anti-flavivirus therapeutics.

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Mevastatin Markedly Potentiates the Anti-HCV Activity of Selective Inhibitors of HCV Replication and Delays or Prevents the Emergence of Antiviral Resistance

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Statinsare 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors that are widely used for the treatment of hypercholesterolemia. Recently, it was reported that certain

statins inhibit HCV RNA replication [Ikeda et al., 2006. Hepatology, 44]. We confirmed the *in vitro* anti-HCV activity of statins and we identified mevastatin as the most potent inhibitor in this series. We studied various combinations of statins with selective HCV inhibitors by performing clearance-rebound assays and antiviral combination assays. We also studied whether mevastatin can delay or prevent the development of resistance against inhibitors of HCV replication. For the clearance-rebound assays, Huh-9-13 replicon containing cells were cultured for six consecutive passages in the presence of a selective HCV inhibitor, alone or in combination with mevastatin and in absence of neomycin selection. During the rebound phase, the inhibitor was removed and cells were cultured for three passages in the presence of neomycin. If antiviral therapy is able to clear the replicon from the culture, the cells will not survive when cultured in the presence of neomycin in the rebound condition. Several antiviral combination assays were performed as described before using Huh-5-2 replicon containing cells [Paeshuyse et al., 2006. Hepatology, 43]. For combination resistance selection, Huh-9-13 replicon containing cells were cultured in the presence of neomycin selection and in the presence of mevastatin or HCV-796, or a combination of mevastatin and HCV-796 at various ratios. Replicon cells that formed visible colonies were selected for further characterization. Neither mevastatin nor the selective HCV inhibitors were able to cure the cells from replicon after six passages of antiviral pressure. However, the combination of mevastatin with polymerase or protease inhibitors resulted in an efficient clearance of the cells from replicon. Combination of mevastatin with IFN-a, with selective HCV polymerase or protease inhibitors elicited an additive anti-HCV effect. Mevastatin was able to decrease (at low concentrations) or prevent (at high concentrations) the emergence of antiviral drug resistance against HCV-796.

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Comparative Virtual and Experimental Medium Throughput Screening for Hepatitis C Virus Polymerase Inhibitors

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Although many search aim at the development of actives antiviral drugs, for most patient infected by one genotype, persistent HCV infection cannot be controlled by antiviral therapy. This has encouraged the search for more potent new antivirals to HCV. Previous work has demonstrated that the NS5B polymerase is a target of choice for multi-therapy programs. Although very promising in vitro, none of the non-nucleoside inhibitors (NNI) discovered so far gives concluding results in the clinic, either because of adverse side effects or little efficacy in patients, due to the rapid selection of mutant virus leading to resistance. Screening campaigns, involving million of compounds issued from several original libraries, are still ongoing with the hope to discover new active compounds. In order to decrease the elevated cost of such research, it is tempting to first virtually screen libraries to select and eventually predict, a list of the best binder molecules that would potentially be inhibitors. The known 3D structure of the polymerase combined to the availability of powerful in silico docking programs allowed such a strategy of antiviral development. In this work, we screened in parallel the same in-house library by an in vitro experimental and an in silico virtual screening. We then compared the results and determined: (i) the best virtual screening protocol and (ii) its predictive power. Our results show that most of our hits are active inhibitors on mutant polymerase harboring the well-known M423T mutation selected by inhibitors targeting the B NNI site. Furthermore, our virtual screening brings an added value to the understanding of the binding mechanism of those hits and some important insight on the potential resistance profile.

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MIV-170, A Novel NNRTI Exhibiting Tight Binding to HIV-1 Reverse Transcriptase (RT)

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The NNRTI MIV-170 has been found to be a very efficient inhibitor of wtHIV and HIV mutant strains resistant to the NNRTIs used in the clinic. To better understand the interaction between MIV-170 and HIV-1 the details of this have been studied by different methods. The kinetics of the interaction between MIV-170 and HIV-1 RT was analysed using a biosensor assay. The association and dissociation rates were determined using immobilized wtRT or RT mutants and MIV-170 as analyte. The results demonstrated that MIV-170 had both a faster association and a slower dissociation rate than efavirenz, nevirapine and delavirdine, thus exhibiting a higher affinity than these compounds. The strength of the interaction between the NNRTIs and RT and RT mutants in the biosensor assay was compared to the reversibility of inhibition in cell culture experiments. In these experiments virus and infected cells were incubated with MIV-170 and other NNRTIs for various times and after removal of the compounds the remaining infectivity was assayed. X-ray analysis of the binding of MIV-170 to HIV-1 RT displayed extensive interactions, not only between the compound and the lining amino acids but also between these residues, turning the binding cavity into a rigid entity and explaining the tight binding in the biosensor assay and the inactivation of HIV.

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