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A structurally biased combinatorial approach for discovering new anti-picornaviral compounds

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Abstract

Background: Picornaviruses comprise a family of small, non-enveloped RNA viruses. A common feature amongst many picornaviruses is a hydrophobic pocket in the core of VP1, one of the viral capsid proteins. The pocket is normally occupied by a mixture of unidentified, fatty acid-like moieties, which can be competed out by a family of capsid-binding, antiviral compounds. Many members of the *Picornaviridae* family are pathogenic to both humans and livestock, yet no adequate therapeutics exist despite over a decade's worth of research in the field. To address this challenge, we developed a strategy for rapid identification of capsid-binding anti-picornaviral ligands. The approach we took involved synthesizing structurally biased combinatorial libraries that had been targeted to the VP1 pocket of poliovirus and rhinovirus. The libraries are screened for candidate ligands with a high throughput mass spectrometry assay.

Results: Using the mass spectrometry assay, we were able to identify eight compounds from a targeted library of 75 compounds. The antiviral activity of these candidates was assessed by (i) measuring the effect on the kinetics of viral uncoating and (ii) the protective effect of each drug in traditional cell-based assays. All eight of the candidates exhibited antiviral activity, but three of them were particularly effective against poliovirus and rhinovirus.

Conclusions: The results illustrate the utility of combining structure-based design with combinatorial chemistry. The success of our approach suggests that assessment of small, targeted libraries, which query specific chemical properties, may be the best strategy for surveying all of chemical space for ideal anti-picornaviral compounds. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Anti-picornaviral; Capsid-binding; High-throughput; Screen

1. Introduction

Picornaviruses comprise a family of small, non-enveloped viruses containing a positive-sense RNA genome encased in a protein capsid (for a review, see [1]). The picornavirus family is subdivided into five genera: the apthoviruses, the cardioviruses, the hepatoviruses, the rhinoviruses and the enteroviruses. The hepatoviruses, rhinoviruses and enteroviruses are responsible for a wide array of human illnesses. The enteroviruses, which include the polioviruses, echoviruses and coxsackieviruses, are associ-ated with poliomyelitis, myocarditis, aseptic meningitis and encephalitis. The rhinoviruses encompass over 100 different serotypes and are responsible for roughly 40% of all common colds. Currently, there are no commercially available drug therapies for any of the diseases caused by picornaviruses.

These viruses share a common icosahedral capsid archi-tecture constructed from 60 copies of four proteins (VP1, VP2, VP3 and VP4) as revealed by crystallographic studies of human coxsackievirus type B3 [2], echovirus 1 [3], po-liovirus type 1 [4], 2 [5] and 3 [6], and rhinovirus types 1A [7], 3 [8], 14 [9] and 16 [10]. In all of these structures the virus surface is punctuated by broad depressions, or can-yons, that separate prominent star-shaped mesas at the five-fold axes and propeller-like features surrounding the three-fold axes of the particle. The canyon has been shown

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to be the site of receptor attachment for major group rhinoviruses and for poliovirus (reviewed in [11]). At the base of the canyon there is an opening into the hydrophobic core of VP1. In most of the entero- and rhinovirus structures solved to date the core of VP1 is filled with a long, thin, worm-like density feature. This ambiguous feature has been dubbed the 'pocket factor' and is generally modeled as sphingosine [6], palmitate [3], or myristate [2], though its true chemical identity remains unknown.

In 1979, investigators at Sterling-Winthrop identified a new antiviral compound, arildone, that bound to the virion of polio and closely related viruses [12]. Since then, a number of antivirals, primarily targeting rhinoviruses, have been developed (for a review, see [13]). Structural studies of virus–drug complexes have shown that these compounds displace the pocket factor and bind in the hydrophobic core of VP1 [5,14–17]. Drug binding has been shown to inhibit infectivity by two different mechanisms, which are not mutually exclusive. The transition to the A particle, which mediates cell entry, requires large-scale changes in the capsid structure, including the loss of VP4 and the exposure of residues 1–80 of VP1, which are internal to the N particle. The stabilization of the virus that occurs upon drug binding has been recently shown to be due to a novel entropic effect imparted by ligand binding [18], and not through rigidification of the capsid as has been previously suggested [13,14]. Secondly, for some rhinoviruses, drug binding induces small local conformational changes in loops at the base of the canyon that prevent receptor attachment [19,20,34]. Dove and Racaniello recently have shown that drug binding (which does not affect receptor binding at physiological temperatures) interferes with poliovirus receptor attachment at 4°C [21]. Since drug binding by poliovirus does not result in significant local structural changes at the base of the canyon [17], these results may suggest that inhibition of receptor attachment also may be attributed to the ability of drugs to inhibit small energy-dependent conformational alterations required for tight receptor binding.

The predominantly hydrophobic nature of the binding site and the unusual mechanism of action of the capsid-binding drugs present a number of challenges to traditional structure-based and other so-called rational drug discovery approaches. However, the wealth of structural data on complexes of these drugs with representative rhino-, polio- and coxsackieviruses [2,4–10] makes this an excellent test system for developing tools to aid in the development of drugs directed at non-enzyme targets. Investigators at Sterling-Winthrop and, more recently, Viropharma have collaborated with Michael Rossmann's laboratory at Purdue to use structures of virus–drug complexes in the late stages drug development. One of the products of this effort, pleconaril, is undergoing clinical trials for the treatment of enterovirus induced encephalitis and for rhinovirus induced colds. We have undertaken an alternative approach, which employs a hybrid of structure-based design into the VP1 binding sites and combinatorial chemistry to develop potential new leads for this family of capsid-binding drugs.

In work that has been published previously, the design was initiated by characterizing the ligand binding site in the hydrophobic core of VP1 using the program MCSS [22]. MCSS produces maps of the preferred binding sites of small molecular fragments by simultaneously subjecting thousands of randomly placed copies of fragments to energy minimization within the force field of a macromolecular target [23]. Fragment maps centered on the VP1 core of the poliovirus and the rhinovirus capsid were calculated for a number of functional groups. These maps immediately suggested a template for a class of ligands which differed significantly from any of the capsid-binding drugs that were published at that time [22]. Limited combinatorial libraries of ligands resembling this template were synthesized and screened using a high-throughput assay [24] in which virus is incubated with crude libraries and the components that bind are identified by mass spectrometry. An initial screen of a prototype library containing 75 compounds identified eight possible candidates. Because the initial screen with the full library was very noisy, potential binders were re-synthesized as members of smaller six compound sub-libraries. These sub-libraries were re-screened with the mass spectrometry assay to confirm the previous results and tested for reduction of the rate constant for uncoating with an immunoprecipitation assay. Promising leads were individually synthesized and also tested for their effect on the rate constant of uncoating and inhibition of in vitro cell lysis. These secondary screens yielded three compounds that inhibit uncoating and infectivity of the Mahoney strain of type 1 (P1/Mahoney) poliovirus with MICs in the 0.1–10 μM range (where the MIC is the minimum concentration required to inhibit viral replication in a tissue culture-based assay). The compound with the highest activity against type 1 poliovirus also had an IC_{50} in the 0.1 μM range when assayed with two different serotypes of rhinoviruses (where the IC_{50} is the concentration required to reduce infectivity by 50%).

2. Results

2.1. Assay development using a radiolabeled anti-picornaviral compound

A diagram of the mass spectrometric screening assay is shown in Fig. 1. The ability of the assay to isolate and detect complexes with ligands whose affinities are at the lower limit of the range for acceptable leads (~1–10 μM) was determined by using ³H-R77975 (Fig. 2A; Janssen Pharmaceutica), which has a MIC of 3.061 μM for P1/Mahoney [17]. P1/Mahoney was incubated overnight at 4°C in a 8×10^{-6} M ³H-R77975 solution (4×10^{-9} mol)
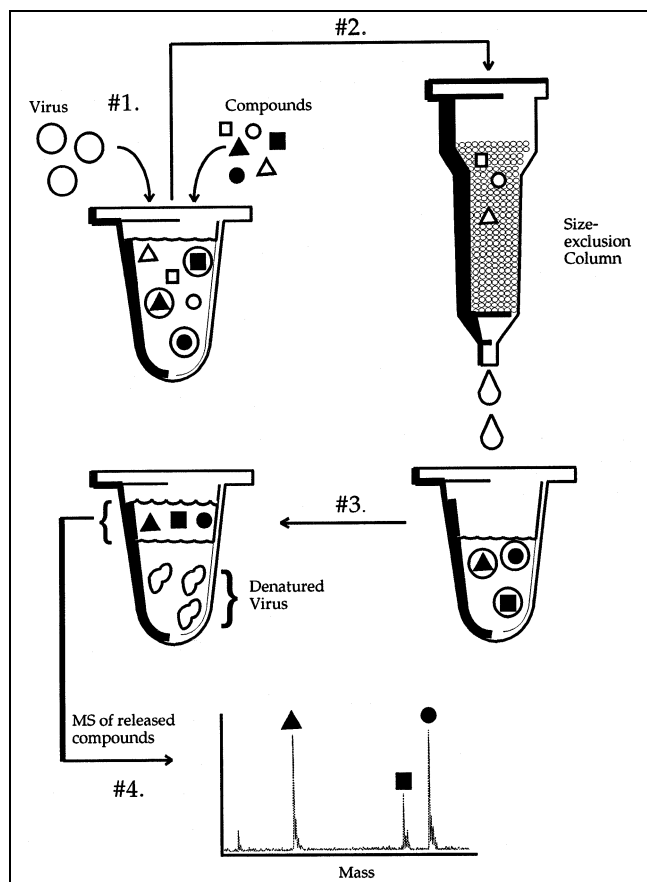
Fig. 1. Schematic of high-throughput mass spectrometry screen. (#1) Virus is incubated with a compound or mixture of compounds. (#2) Since the virus molecular mass (8.5×10^{-6} Da) is much greater than that of the compounds tested, compounds unbound to the virus are easily separated from virus–compound complexes by size exclusion chromatography. (#3) The complexes are then denatured with an organic solvent, which separates the hydrophobic compounds from the virus into an organic phase. (#4) The organic phase is dried down and the residue analyzed by mass spectrometry.
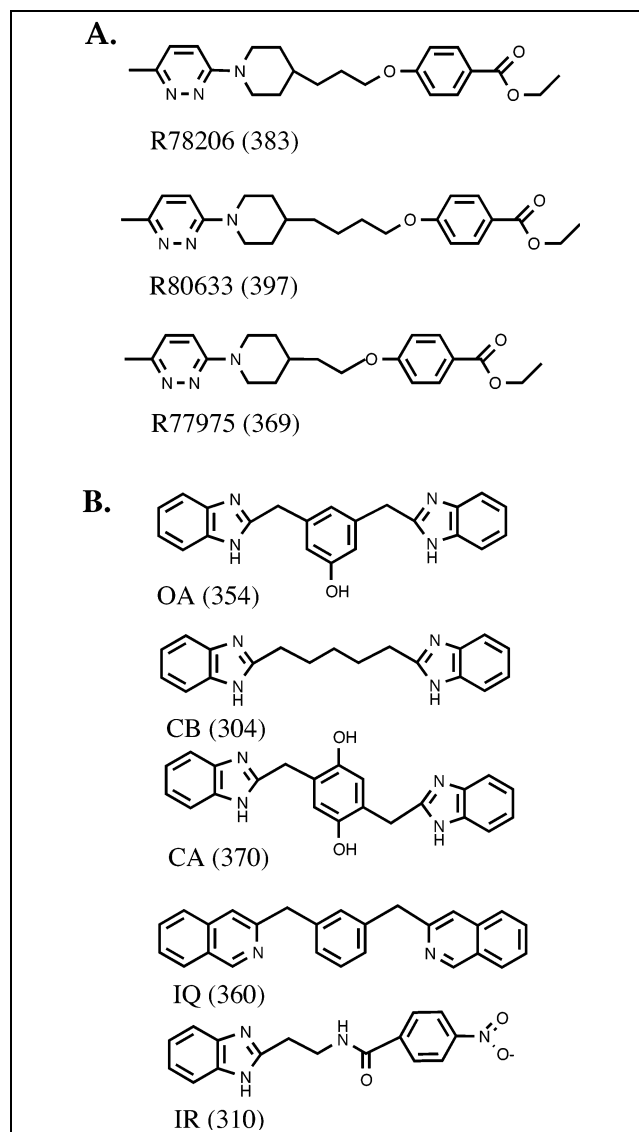

Fig. 2. Schematic of compounds. (A) Janssen Pharmaceutica compounds. (B) Structurally related analogs of Janssen compounds thought to have no affinity for P1/Mahoney by prior crystallographic analysis. Molecular masses are given in Da in parentheses.

with a VP1 pocket:compound ratio of 1:1. Virus–drug complexes were isolated by low pressure size exclusion chromatography on a Sephacryl S-300 column (8.5 cm length, 1.3 cm diameter). The void volume, containing virus and presumably drug, was collected in fractions 8–10. The passage of ^{3}H-R77975 through the column was monitored by scintillation counting of aliquots from

each fraction. The amount of ^{3}H-R77975 that eluted with the virus amounted to roughly 33% of the counts incubated with the virus (Table 1, column A). The remainder of the counts eluted as a broad peak, suggesting that a

Table 1
Assessing assay yield and selectivity with a radiolabeled ligand

	A ^{3}H-R77975+PV (%)	B ^{3}H-R77975 only (%)	C ^{3}H-R77975+R78206+PV (%)
Incubation cpm	100	100	100
Void volume cpm	33	0	0
Extracted cpm	28	0	0
CPM after drying and re-suspending in AcN	24	0	0

For all columns, 100% represents the total number of counts used in the incubation and each value given below is a percentage of the initial cpm. A: P1/Mahoney incubated with ^{3}H-R77975. B: No ^{3}H-R77975 is recovered in the void volume if there is no virus in the incubation. C: In the presence of R78206, a more potent inhibitor than R77975, no counts are recovered in the void volume.
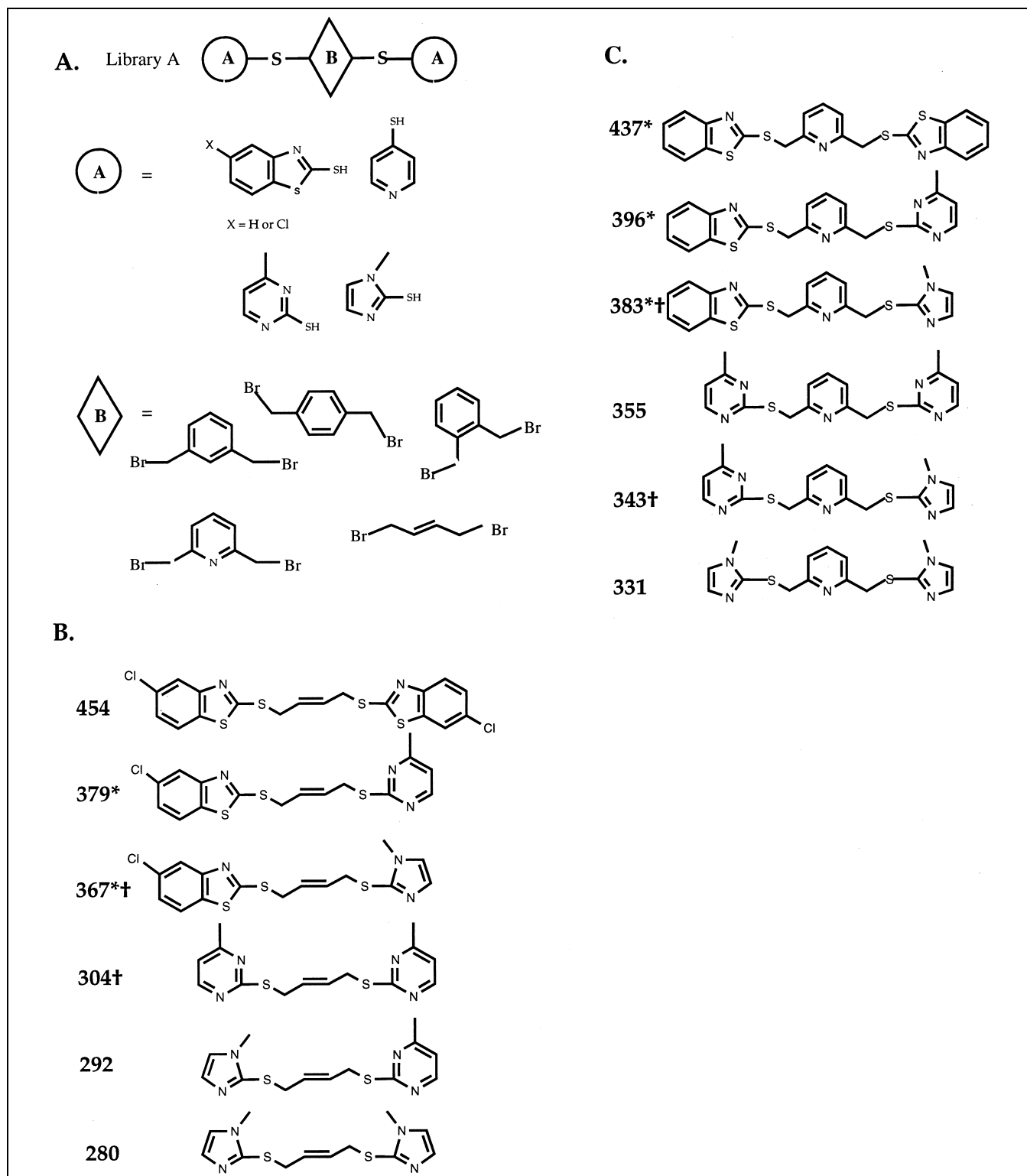
Fig. 3. Schematic of libraries. (A) Schematic of library A. (B) Sub-library 6.1. (C) Sub-library 6.2. Each compound in (B) and (C) is identified by its corresponding mass. Compounds denoted † were initially identified in our library A screens. When each sub-library was tested with the mass spectrometry assay, all compounds marked with an asterisk (*) were found to be associated with virus.

significant fraction of the bound drug dissociated during the course of separation (data not shown). This is consistent with the expectation that the off-rate of this poorly binding ligand is high. The peak fractions from the void volume peak were pooled. Two volumes of ethyl acetate were added and the mixture vortexed in order to denature the virus and partition ^{3}H-R77975 into an organic phase for an easy one-step purification. After the ethyl acetate

layer was removed and transferred to a fresh Eppendorf tube, 10% of this material was removed and the cpm determined. The extraction step recovered an estimated 85% of the material present in the original peak. After vacuum drying the ethyl acetate away and re-suspending the residue in acetonitrile (AcN), roughly 80% of the extracted material was recovered. When no virus was used in the incubation, the amount of counts in the void volume corresponded to the background (Table 1, column B).

Selectivity of the assay was shown by a competition experiment (Table 1, column C) using R78206 (Fig. 2A), which has a MIC of 8 nM [17], nearly a 1000-fold greater affinity for P1/Mahoney than R77975. In this control experiment, equal concentrations of ^{3}H-R77975 and R78206 (8 µM, corresponding to 2×10^{-10} mol of each drug in the assay) were incubated with virus (2×10^{-11} mol binding sites). As expected the presence of the higher affinity ligand (R78206) at a 10-fold excess over available sites resulted in the exclusion of the labeled low affinity ligand (R77975).

In order to avoid the dissociation of weakly binding ligands during the development of the low pressure size exclusion separation of virus–drug complexes, all subsequent experiments were performed using a spin column (BioRad), which allows recovery of the void volume within 4 min.

2.2. Mass spectrometry screenings of R78206, R77975 and R80633 incubated with virus

Before using the assay to identify ligands in experimental combinatorial libraries (Fig. 3) several control experiments were performed using mixtures of Janssen compounds. To show that the assay was capable of isolating multiple compounds, three Janssen compounds, R77975, R80633 and R78206 (Fig. 2A), were incubated with virus to allow binding. The results of this assay are shown in Fig. 4A. All three compounds are present in equimolar amounts (concentration 4 µM which corresponds to
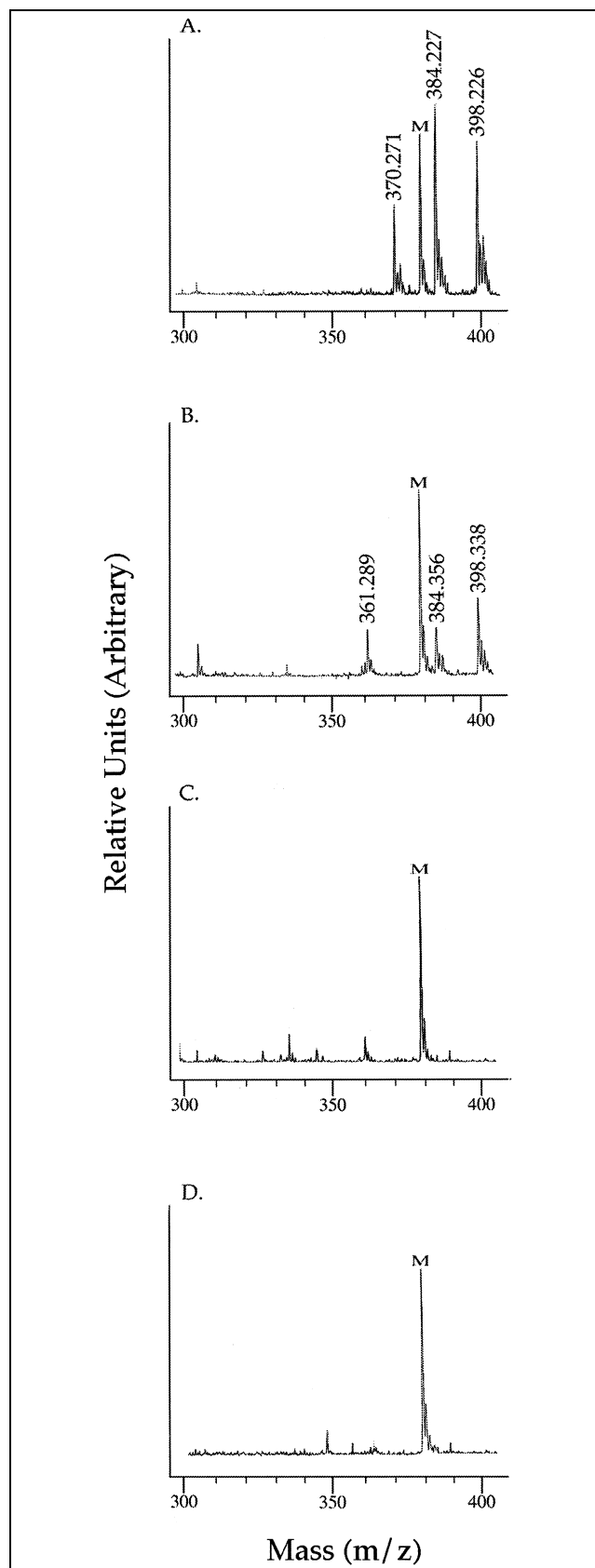

Fig. 4. Validation of mass spectrometry assay. (A) Mass spectrum illustrating that R77975, R78206 and R80633 are recovered from our poliovirus binding assay. (B) Mass spectrum showing that the binding assay is selective. In this experiment, the virus had been incubated with R78206, R80633 and five other compounds with no known ability to bind poliovirus as judged from previous crystallographic assays (Fig. 2B). IQ may be a weak binder as suggested by our assay. (C) R77975, R78206 and R80633 incubated without virus. The absence of virus in the incubation prevents the low molecular mass compounds from appearing in the void volume. (D) An incubation with virus only. No mass peaks for any of the tested compounds are visible. M corresponds to one of the α-cyano-4-hydrocinnamic acid matrix peaks used in these assays. Its [M+H]$^+$ peak is 379.4. The molecular masses of the tested compounds are: R77975 = 369, R78206 = 383, R80633 = 397, IQ = 360, IR 310, CA = 370, CB = 304 and OA = 354.

2×10^{-9} mol of each compound), and the virus concentration was set such that there were exactly enough sites to bind the total number of compounds. R80633 has a MIC of 511 nM against P1/Mahoney [17]. After an overnight incubation of virus and compounds at 4°C, the compounds were put through our assay, and the resulting matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF) mass spectrum contained only three peaks corresponding to the three compounds. The control experiment (Fig. 4C) without virus in the incubation gave no indication of R77975, R78206 or R80633. An additional control in which only virus and no compounds were present in the incubation was also screened, giving no signal (Fig. 4D).

2.3. Mass spectrometry screenings of R78206, R80633 and five other compounds incubated with virus

To verify that the binding of drug to virus was specific, we constructed a test library containing two compounds with proven antiviral activity, R78206 and R80633, alongside a cocktail of five structurally related compounds (Fig. 2B) with different molecular weights and with no expected ability to bind virus (unpublished crystallographic data). In this incubation, each compound was present in an equimolar amount (4×10^{-9} mol at a concentration of 5 μM), and there were exactly enough binding sites to bind all of the compounds. The MALDI-TOF spectrum from this experiment indicated the expected peaks for R78206 and R80633 (Fig. 4B). Of the other five compounds, a peak was detected for IQ, indicating that it may be a weak binder. All other molecular mass ion peaks for the other compounds were absent.

2.4. Mass spectrometry screening of library A incubated with virus

A library of 75 potential compounds was synthesized using combinatorial chemistry (Fig. 3A). The design of this library is a modification of a previously published design based on computer modeling studies on the VP1 pocket of poliovirus and rhinovirus [22]. A 500 μl incubation was set up such that the moles of the library compounds were 15-fold greater than the moles of virus binding sites. Specifically, the concentration of library was 3.24×10^{-4} M in the incubation for a total of 1.62×10^{-7} mol of compounds, while the concentration of virus sites was 2.16×10^{-5} M. Given these amounts, there are enough sites to bind up to approximately five compounds, assuming an equal yield of each library compound. This incubation was run through the mass spectroscopic assay (Fig. 1) yielding the spectrum in Fig. 5A.

A second screening of library A was done under different conditions to examine the ability of the assay to select the same candidates. This incubation used a 10:1 ratio of compound to binding sites and the concentration of li-
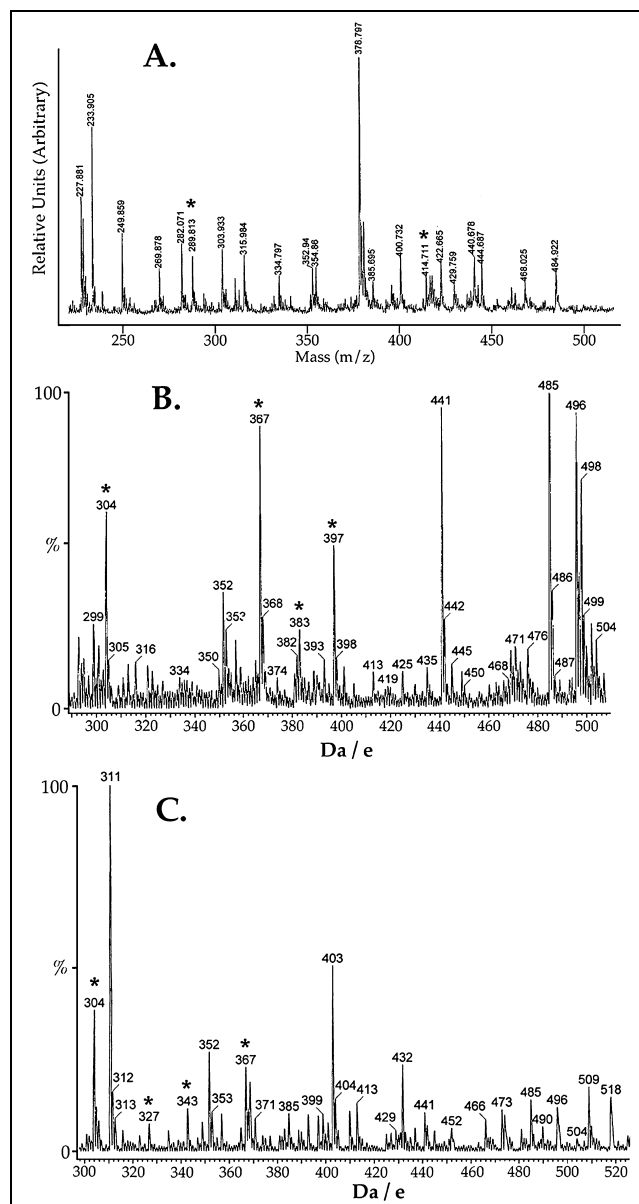

Fig. 5. Mass spectrometry screen of library A. (A) MALDI-TOF spectrum of library A screened with P1/M. An α-cyano-4-hydrocinnamic acid matrix peak is denoted M. When library A was re-screened using electrospray mass analysis, the extracted material was first re-suspended in methanol, which generated a precipitate. A spectrum of the supernatant was taken and shown in (B). The precipitate was re-suspended in ethanol and its mass spectrum is shown in (C). Mass peaks corresponding to compounds in the library are marked with asterisks.

brary in the incubation was 1.2×10^{-4} M. After the sample was dried to completion, it was re-suspended in 50 μl of methanol. Upon addition of methanol, a white precipitate formed. This material was precipitated by centrifugation at room temperature for 10 min at $17\,000 \times g$. The supernatant was separated, while the pellet easily re-suspended in 50 μl of ethanol. Both samples were analyzed by electrospray mass spectrometry.

The results from both screens are shown in Fig. 5B,C. The masses range of compounds in library A range from

274 to 506 Da, but the spectra have been truncated for the sake of clarity. All of the mass peaks which correspond to compounds in library A are marked with an asterisk. In all cases, there are a large number of peaks that cannot be assigned to any compound in our libraries. These extraneous peaks may represent either impurities in the reagents used or small molecules (e.g. polyamines) that are normally associated with the virus. The peaks may also simply represent noise in the spectra, which is being produced near the sensitivity threshold of the available instrument due to limitations in the amount of virus that it is practical to use in a given experiment. Fortunately, the identity of the extraneous peaks varies from assay to assay and, in some cases, from shot to shot with a given sample, whereas the peaks we have interpreted as true signal are reproducible. Despite the great deal of noise, the MALDI-TOF spectrum (Fig. 5A) consistently shows two peaks

corresponding to molecular masses of compounds in our library. These are 289 and 414. The mass spectrum of the precipitate (Fig. 5C) used in electrospray analysis yielded four relatively strong peaks, corresponding to molecular masses 304, 327, 343 and 367. The mass spectrum of the supernatant (Fig. 5B) used in the electrospray analysis confirmed 304 and 367, and added two other candidate ligands, 383 and 397. In total, eight unique peaks were identified from all spectra that could correspond to library compounds. We attribute the differences between the three assays shown in Fig. 5 to differences in sample handling (e.g. differential solubility in the solvents used to prepare the extracted ligand for mass spectrometry) and to differences in the mechanisms for volatilizing samples in the two mass spectrometry technologies. These differences are exacerbated by the fact that the assays are being run near the sensitivity threshold of the available instruments and could probably be overcome by using much larger amounts of the virus in the samples.

2.5. Mass spectrometry screening of sub-library 6.1 incubated with virus

In order to improve the signal and to verify our results from the library A screen, we synthesized a small combinatorial library (containing six compounds) that included two of the compounds (304 and 367) that were identified in the electrospray spectra (Fig. 2B). As shown in Fig. 6A, screening of this library with the assay only re-produced peak 367 from the library A screen. The absence of the 304 peak may indicate that its presence in the library A screen was a false positive. Individual synthesis and screening of 304 did not give a signal in our assay (data not shown). Individual synthesis of 367 and a subsequent re-analysis of 367 with P1/Mahoney returned the signal (data not shown).

2.6. Mass spectrometry screening of sub-library 6.2 incubated with virus

Since the initial modeling work [22] suggested that the central region of the pocket was capable of accepting larger aromatic groups, another six compound library was synthesized with the same terminal groups as in sub-library 6.1, but with the aliphatic linker being replaced with a linker containing an aromatic ring (Fig. 2C). This library includes two additional compounds (383 and 396) that were identified in the original electrospray screen of library A. As indicated only the larger compounds in sub-library 6.2 bound to the virus (Fig. 6B). As with the sub-library 6.1 screen, one of the candidates, 343, appeared to be a false positive.

2.7. Rate constants of 160S to 135S conversion

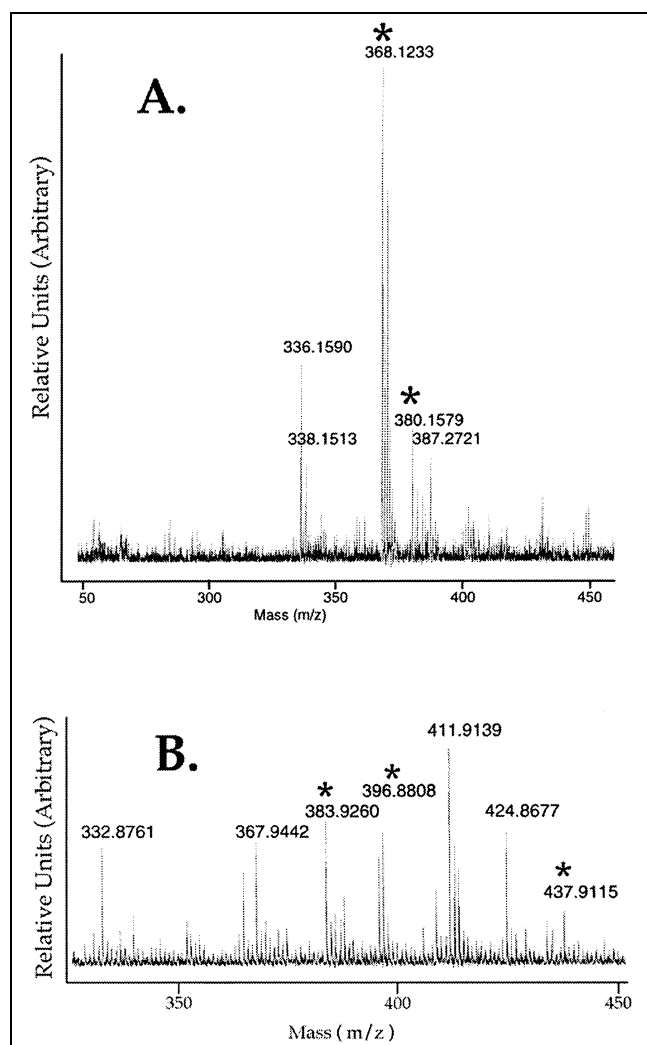We have previously shown that known capsid-binding

Fig. 6. MALDI-TOF mass spectrometry screen of sub-libraries. Sub-library 6.1 (A) and sub-library 6.2 (B) screened with P1/Mahoney. Mass peaks in the spectra corresponding to compounds in the sub-libraries are marked with asterisks. The matrix compound used in this experiment was DHB, which does not produce characteristic peaks in the mass range displayed.

A.

B.

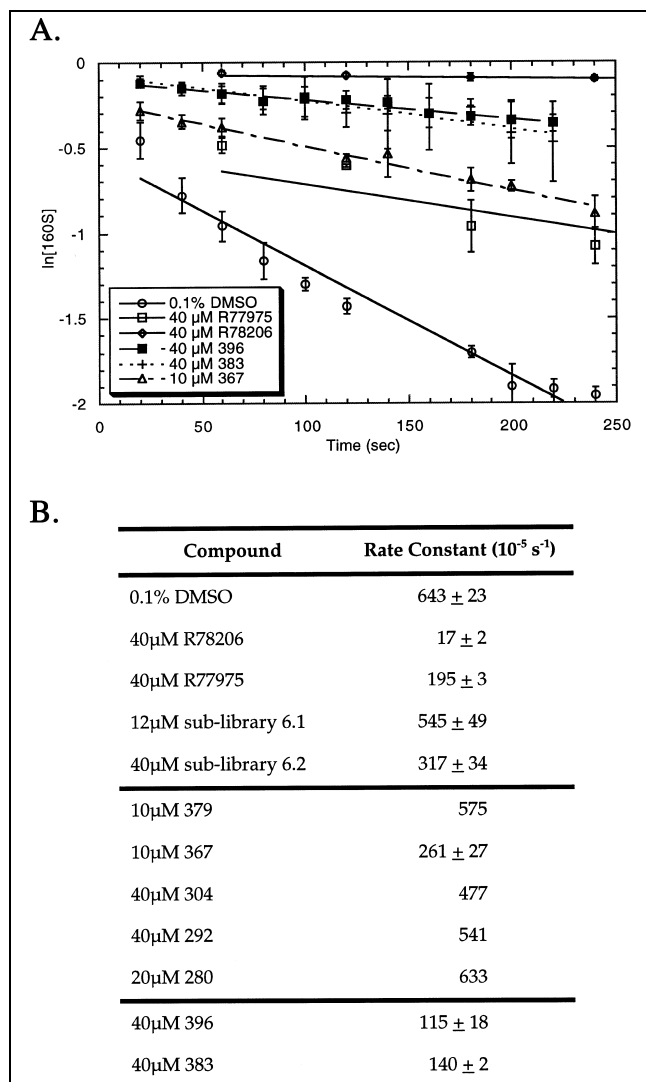| Compound	Rate Constant (10^{-5} s^{-1})
0.1% DMSO	643 ± 23
40µM R78206	17 ± 2
40µM R77975	195 ± 3
12µM sub-library 6.1	545 ± 49
40µM sub-library 6.2	317 ± 34
10µM 379	575
10µM 367	261 ± 27
40µM 304	477
40µM 292	541
20µM 280	633
40µM 396	115 ± 18
40µM 383	140 ± 2

Fig. 7. Rate constants for the 160S to 135S transition in the presence of various compounds at 43°C. (A) Summary of rate constant plots. The data for R77975 extends to 7 min, but the plot has been truncated for the sake of clarity. (B) Table of rate constants for the compounds tested at 43°C.

agents slow the rate of thermally induced 160s to 135S conversion, and that there is a rough correlation between the rate of conversion at a given temperature and the MIC for the compound [18]. Determination of the rate constant for this process can be calculated by immunoprecipitating radiolabeled 135S particle as it is formed in vitro as a function of time. Where possible the compound concentration was arbitrarily set at 40 µM in conversion buffer containing 0.1% dimethyl sulfoxide (DMSO). However, some of the compounds or libraries, were not soluble to that degree, so the concentrations listed in Fig. 7B represent nearly saturated solutions. Note also that the concentrations used are based on the mass of the dried oil and are therefore very approximate and represent an upper limit to the true concentration.

Fig. 7 shows the rate constants for the 160S to 135S

transition at 43°C for ^{3}H-P1/Mahoney which had been pre-incubated with sub-library 6.1, sub-library 6.2, 280, 292, 304, 367, or 379, 383 and 396. Rates for virus alone in 0.1% DMSO and for virus pre-incubated with R78206 and R77975 are shown as controls. We previously have shown that 0.1% DMSO has no effect on the rate constant for the process, whereas higher concentrations can inhibit the transition (data not shown). Library 6.1 and compound 304 showed a marginally significant reduction in the rate of conversion. The low level of protection afforded by library 6.1 may reflect the low total drug concentration used in the assay due to solubility limitations. Library 6.2 and three of the pure individually synthesized compounds (367, 383 and 396) showed a significant reduction in the rate of conversion. The rate reduction observed for 367 was comparable to that observed for R77975, even though a much lower level of 367 was used due to solubility limitations. The rate reduction observed for 383 and 396 were intermediate between those observed for R77975 (whose MIC for P1/Mahoney is 3 µM) and R78206 (whose MIC for P1/Mahoney is 0.008 µM).

2.8. Cell-based assays

To verify that the candidates from our screens might protect cells from invasion by picornaviruses, we attempted to determine the minimum inhibitory concentrations of the compounds. When tested against P1/Mahoney in a HeLa cell-based cytopathic effect assay, the potency of the three candidates, 367, 383 and 396, appeared to correlate well to their rate constants (Table 2). None of the other compounds tested exhibit any activity against poliovirus at concentrations up to 25 µM. Six of the individually synthesized compounds (304, 355, 367, 379, 383 and 396) were tested in Viropharma's automated infectivity assay against a variety of rhino- and enteroviruses

Table 2
Minimum inhibitory concentrations of candidates

(A) MIC versus poliovirus 1/Mahoney	
Compound	MIC (µM)
R78206	0.008[a]
R80366	0.511[a]
R77975	3.061[a]
396	11
383	0.26
367	3.2

(B) Human rhinoviruses		
Compound	HRV type	IC_{50} (µM)
396	14	no activity
383	14	0.8
383	3	0.55
379	14	3.2
367	14	no activity

(A) MICs against poliovirus type 1. (B) IC_{50} against rhinovirus.
[a]These values were previously presented in [17].

(HRV1A, 3, 14, 16, polio 1, 2 and 3 (Sabin strains), cox-sackievirus B3 (Gauntt strain) and echovirus 30 (Bastianni strain)). Compounds 304 and 355 had been submitted as negative controls. Two of the compounds (379 and 383) tested positive for HRV3 and 14. Curiously, the three compounds that reproducibly showed activity against the Mahoney strain of type 1 poliovirus in our thermal conversion and infectivity assays failed to show activity against the Sabin strain of any of the three serotypes of poliovirus in the Viropharma assay. This may be due to differences between the closely related Sabin and Mahoney strains of type 1 poliovirus. Compound 383 was found to be slightly toxic at 25 µM, but this concentration is $> 10 \times$ the IC_{50} for P1/Mahoney, HRV3 and HRV14.

3. Discussion

Despite advances in structure-based design methods and the vast ligand diversity provided by combinatorial chemistry, rational drug development remains an immature and imprecise art. Indeed, the successful applications of structure-based design approaches to date generally have been restricted to developing existing leads identified by conventional methods and have largely focused on enzyme targets. The arguably more difficult goal of utilizing structure to discover new lead compounds or to develop drugs against targets that are not enzymes is far less well developed. Given the large (and increasing) number of high resolution structures of appropriate targets, we believe the limited success of the structure-based methods is attributable to several factors, including inherent limitations in the accuracy of even the best structures, the lack of general computational approaches to predict the free energy of binding, and the paucity of tools to facilitate the translation of structure into synthesizable ligands. In contrast, combinatorial chemistry has been typically used to identify leads that are developed by conventional SAR methods. However, the synthesis of highly diverse combinatorial libraries, has proven more challenging than many had predicted. Moreover, the vast majority of diversity of libraries is wasteful for any given target, especially when other information is available, e.g. a structure of the target, a lead or a known enzyme mechanism. Wasted diversity places unnecessary demands on synthetic methods and particularly on the screening assays, because noise contributed by the plethora of weak binders in a library can mask the signal of strong binders. The complementarity between the strengths and weaknesses of structure-based and combinatorial approaches immediately suggests the utility of a hybrid approach, in which the structure of a target is used to develop structurally biased combinatorial libraries whose focused diversity in 'regions of chemical space' are likely to be productive.

We have used such a hybrid approach to design new leads in a family of antiviral drugs that inhibit replication of enteroviruses and rhinoviruses by binding to the virions and inhibiting conformational changes that are required for receptor binding or cell entry. Although several members of this family of antivirals have been described previously and structures of virus–drug complexes were available prior to this work, the design did not make explicit use of the known drugs or virus–drug complexes, with the exception of using the complexes to define the binding site in the virus. Indeed, in retrospect, one could argue that prior knowledge of the binding site was not necessary based on three observations: (i) the binding site is occupied by a natural ligand (or pocket factor) in the structures of most rhino- and enteroviruses; (ii) genetic studies in poliovirus have demonstrated that the ligand binding site is important in regulating virus stability and cell entry [25,26] and (iii) simple modeling demonstrates that the natural ligand does not optimally fill the binding site, suggesting that larger ligands might bind more strongly.

We have used fragment binding maps generated by the program MCSS to develop a general template that has served as a structural bias for limited combinatorial libraries of compounds. A library containing 75 compounds has been characterized using a high throughput mass spectrometric assay capable of identifying ligands from crude libraries that bind virus. The screens identified three new anti-picornaviral compounds. Two of these leads possess large aromatic groups in the center of the molecule, making them very different in structure from previously described active antivirals at the time that these studies were initiated (see below). The selection of three leads from this library with micromolar and submicromolar activity represents a highly respectable 10% success rate.

The activity of our leads was confirmed by a rapid kinetic assay and a traditional cytopathic inhibition assay. While the ordering of MICs and degree of rate constant inhibition does not directly correlate, the difference in rate constant inhibition between active compounds and poorly or non-active compounds is readily apparent (Fig. 7). Our data suggest that the IP assay provides good predictive values in a shorter time scale than the common cell-based assay. Moreover, because the virus is generally less sensitive to solvent and impurities (including unreacted starting materials) than cultured cells, the IP assay can be performed with crude libraries, eliminating the need for rigorous purification at early steps in the process. The combined screens identified three compounds (367, 383 and 396) that are active against the Mahoney strain of type 1 poliovirus. All of these compounds were micromolar inhibitors, with MICs of 11, 3.2 and 0.26 µM for 396, 367 and 383, respectively. In an automated screen versus a range of rhino- and enteroviruses, compound 383 also was shown to be active versus rhinovirus 3 ($IC_{50} = 0.55$ µM) and rhinovirus 14 ($IC_{50} = 0.8$ µM), and another compound (379) was shown to be active versus rhinovirus 14 ($IC_{50} = 3.2$ µM). We have solved the structures of com-

plexes of P1/Mahoney and two of our candidate drugs (367 and 396) [27]. Interestingly, although both compounds bound in the VP1 pocket, low contour maps of the P1/M–367 complex revealed an alternate and significantly different binding mode from those previously reported. We are presently attempting to use this information in our design efforts.

The synthetic approach used in the generation of our pilot libraries is quite robust and could easily be used to create larger libraries of compounds from readily available and inexpensive starting reagents. Such libraries could be expected to generate additional ligands, some of which may have significantly better MIC's or a broader range that any of those identified to date. Indeed, after our work using this strategy began, Schering-Plough published a compound that could be synthesized using our approach, having a ~ 10 nM MIC against polio and micromolar or submicromolar activity against a broad range of enterovirus [28–30].

One of the major problems limiting the size of libraries that can be screened is the noise level of the mass spectrometric assay. Even with the present state-of-the-art mass spectrometers, the noise level would preclude screening libraries much larger than 100–200 compounds in the assay as presently formatted. Although this could be alleviated by using larger amounts of virus in the assay, this would ultimately become prohibitively expensive. An alternative approach that is suggested by our subscreening small 6–10 compound libraries would be to synthesize a number of small libraries, each of which asked a 'question' about optimal components for the 'head groups' and 'linkers'. Such libraries could be then screened in small groups and the information gained from each group used in the design of the next round of small libraries. These groups of sub-libraries could be considered as components of a sparse matrix or limited factorial screen that could be analyzed by standard data mining approaches. This structurally biased factorial approach could be used to efficiently sample a large volume of the entire 'chemical space' than would be feasible with the full combinatorial approach.

4. Significance

We have developed and utilized a rapid, high-throughput mass spectrometry assay for the identification of new broad spectrum anti-picornaviral compounds from combinatorial libraries. The design of our test library was guided by structure-based modeling into the VP1 pocket of both P3/Sabin poliovirus and rhinovirus 14. Candidates identified in the mass spectrometry assay were verified by synthesizing smaller sub-libraries containing the candidates and re-screening with the assay. Verified candidates were tested for inhibition of the rate constant for viral uncoating using an immunoprecipitation assay. These results were compared against the MIC from traditional cell-based assays. Using this procedure we isolated eight potential candidates from a 75 compound library. Of these eight, the field was narrowed to three, and the most promising lead appears to have a structure unlike previously described inhibitors. These results demonstrate the utility of structurally biased combinatorial approaches to ligand design.

5. Materials and methods

5.1. Growth, propagation and purification of virus

P1/Mahoney was grown in HeLa cells grown in suspension and purified by differential centrifugation and CsCl density gradient fractionation as described previously [31]. To label the virus with ^{3}H-leucine, a protocol similar to that published earlier [32] was used. Purified virus was dialyzed into phosphate-buffered saline (PBS) and concentrated to 5 mg/ml or greater by ultrafiltration.

5.2. Synthesis of a large (bis)thioether library (library A)

One equivalent of equimolar amounts of the nucleophilic thiophenols (Fig. 3) was dissolved in dimethylformamide (DMF) under dry nitrogen. Ten equivalents of potassium carbonate were added as a solid, and the solution was stirred at room temperature for 5 min. To the resulting mixture was added half an equivalent of an equimolar solution of the allyl- or aryl-dibromide building blocks in DMF. The reaction mixture was stirred at room temperature for 24 h. Insoluble salts were removed by filtration, and the solution was concentrated to dryness at reduced pressure. The residue was partitioned between ethyl acetate and saturated aqueous sodium bicarbonate to remove unreacted starting materials. The organic phase was further washed with water and saturated aqueous sodium chloride, dried over magnesium sulfate, and concentrated under vacuum. The resulting brown oil was decolorized with Norit A as a solution in 1:1 chloroform:methanol and redried.

5.3. Synthesis of sub-libraries 6.1 and 6.2

One equivalent of equimolar amounts of the nucleophilic thiophenols was dissolved in DMF under dry nitrogen. Ten equivalents of potassium carbonate was added as a solid, and the solution was stirred at room temperature for 5 min. To the resulting mixture was added half an equivalent of an equimolar solution of the dibromide building blocks in DMF. The reaction mixture was stirred at 60°C for 24 h. The mixture was then diluted in water and extracted three times with methylene chloride. The organic layers were combined and washed three times with 0.1 N sodium hydroxide, dried with potassium sulfate and concentrated under vacuum. The resulting brown oil was decolorized with activated charcoal as a solution in boiling ethanol and filtered through a bed of Celite. Concentration under vacuum gave a light brown oil.

5.4. Synthesis of single compounds

Reaction conditions were the same as for the large and small bis-thioether libraries. After working up, washing, drying and concentrating as described in Section 5.3, the desired compound was isolated by silica gel chromatography. Compound identity was confirmed by proton nuclear magnetic resonance and the presence of molecular ion peak in fast atom bombardment mass spectra.

5.5. Formation of virus–compound complexes for mass spectral analysis

One hundred μg of virus was incubated overnight at 4°C with a single compound or mixture of compounds in a 500 μl low-binding microcentrifuge tube (Marsh Biomedical). Antiviral compounds were supplied as powders or oils and concentrated stock solutions were made in DMSO. Prior to incubation with virus, aliquots of the libraries were assayed by mass spectrometry to confirm their composition. With the exception of the parent 75 compound library, where only a subset (43%) of the possible compounds were in the library at detectable levels, all of the libraries were shown to be complete. The stocks were diluted into conversion buffer (10 mM HEPES pH7.5, 2 mM $CaCl_2$, 0.1% Triton X-100). The total incubation volume was 75 μl and the DMSO content in the incubations was always 5%. The compound to pocket ratio in the incubations ranged from 1:1 to 333:1. The concentration of compounds in these incubations varied from 1 μM to 2 mM.

5.6. Purification of compounds bound to virus (Fig. 1)

The virus–compound incubations were loaded onto Bio-Spin columns (BioRad) with either a 6 or 30 kDa molecular mass cut-off, which had been equilibrated in water to eliminate salts, which can interfere with mass analysis. The columns were spun at $2000 \times g$ in a Beckman J-6B centrifuge with a swinging bucket rotor at room temperature. The filtrate containing the virus was transferred to a low-binding microcentrifuge tube. Subsequently, 200 μl of ethyl acetate (Sigma) was added to the filtrate. Each sample was vortexed for 30 s, and the emulsification was removed by spinning each sample at $16\,000 \times g$ for 5 min at room temperature. The ethyl acetate organic solvent has a dual purpose: to denature the virus, thereby liberating any virus-bound compound, and to partition the hydrophobic compounds into the organic phase. After spinning, the organic phase was transferred to a new low-binding microcentrifuge tube. The compound-enriched ethyl acetate phase was dried down in a centrivap (Labconco) with heat.

5.7. MALDI-TOF analysis of compounds

To each tube containing the dried compounds, 5 μl of 70% AcN 0.1% trifluoroacetic acid (TFA) was added and vortexed well to re-suspend the compounds. One μl of this solution was spotted onto a sample planchette with 1 μl of matrix solution (see below). The remainder of the sample was re-dried and re-suspended in 1 μl of 70% AcN 0.1% TFA and spotted with 1 μl of matrix solution. Samples were allowed to crystallize at room temperature. The crystalline samples were redissolved on the sample planchette with the addition of 0.5 μl of 70% AcN 0.1% TFA to create a more homogeneous sample spot.

Our initial experiments involved using a matrix of saturated sinapinic or α-cyano-4-hydrocinnamic acid (Sigma) in 70% AcN 0.1% TFA. However, since the matrix peaks for sinapinic acid tended to interfere with those of our compounds, we switched to 30 mg/ml 2,5-dihydroxybenzoic acid (DHB) (Sigma) also dissolved in 70% AcN 0.1% TFA.

Samples were analyzed on a Voyager-DE STR MALDI-TOF mass spectrometer from Perceptive Biosystems (Framingham, MA, USA) in reflectron mode. For every session prior to analysis, the mass spectrometer was calibrated to a bradykinin mass standard (904.4) and one of the matrix peaks, either 225.1 for sinapinic acid or 154.0 for DHB. In MALDI-TOF spectra, the value of each peak is one greater than the molecular mass of the corresponding analyte, because each molecule is ionized in the process.

5.8. Electrospray mass analysis of compounds

After the material was screened and dried down, the residue was re-suspended in either methanol or ethanol, two solvents used commonly in electrospray mass spectrometry. Samples were analyzed on a Micromass spectrometer (UK). Unlike MALDI-TOF, peaks in electrospray mass spectra correspond directly to the analyte's true mass.

5.9. Formation of virus–compound complexes for kinetic experiments

In a 22.5 μl volume, 11 μg of ^{3}H-P1/Mahoney was incubated in a 10–40 μM solution of a single compound or small library. The DMSO concentration in these incubations did not exceed 0.1%, which is known to have no affect on the rate of uncoating (unpublished results). The incubations were brought up to volume using conversion buffer. These incubations were performed overnight at 4°C in low-binding microcentrifuge tubes.

5.10. Immunoprecipitation of 135S

The rate of thermal induced 160S to 135S conversion of virus drug complexes was determined as described elsewhere [18]. Briefly 980 μl of conversion buffer in a 1.5 ml Eppendorf tube was pre-warmed to the specified temperature in a water bath (Isotemp Refrigerated Circulator Model 9100, Fisher Scientific). The 980 μl of buffer contained the compound or library of interest at the same concentration as in the overnight incubation. The temperature was monitored by inserting a thermocoupler probe (Omega) into a 1.5 ml Eppendorf tube which had 1 ml of conversion buffer. When the temperature had stabilized for at least 5 min the experiment was allowed to proceed.

Prior to use, the virus–compound incubation was equilibrated

at room temperature by letting it sit on the bench top for at least 10 min. Since the 135S particle is very hydrophobic, minimizing the degree of loss of 135S to non-specific binding required using siliconized tips (VWR) on all solutions potentially containing the 135S particle. Twenty μl of the virus–compound incubation was added to the pre-warmed buffer and the entire reaction tube was removed from the water bath and vortexed vigorously and returned to the water bath within 3 s. At the appropriate time interval, an 80 μl aliquot was removed and transferred to a low-binding microcentrifuge tube containing 50 μl of ice-cold PBS+buffer (PBS, 1% Triton X-100, 0.1% SDS, 0.5 mg/ml bovine serum albumin, 0.01% NaN_3). The purpose of the chilled buffer is to rapidly bring down the temperature of the aliquot to stop the conversion of native virus into the 135S form.

Thirty-five μl of P1 monoclonal antibody was added to each aliquot. This antibody binds residues 24–40 of the N-terminal end of VP1 which is internal in the native virus but has been shown to be exposed upon the 160S to 135S transition [33]. The amount of antibody required to pull down all of the A particle in an aliquot assuming complete conversion had been pre-determined by standard curve. The incubation was carried out at room temperature for 1 h to allow immune complexes to form. Subsequently, 40 μl of protein A Sepharose CL-4B (Pharmacia) was added to each tube. The tubes were shaken to keep the protein A beads in suspension for 2 h at 4°C. The beads were washed 3–4 times with 300 μl PBS+buffer. After each wash, the beads were pelleted by centifugation at $16\,000\times g$ for 5 min at room temperature. The washes were combined and the pooled wash and the beads were transferred to separate vials containing 7 ml of scintillation fluid (EcoScintA, national diagnostics). The counts in the pellet and supernatant fractions were determined by scintillation counting (LS500TD, Beckman).

5.11. Determination of rate constants

The percentage of 160S at each time point was calculated by using:

$$(\text{total cpm} - \text{bead cpm})/\text{total cpm}$$

where the total cpm is the sum of the counts per minute (cpm) of the washes and bead cpm is the cpm of the beads. The first order rate constants for the conversion were determined by determining the slope of the plot of the log of the percent remaining 160S at each time point versus time using Kaleidagraph 3.0 (Abelbeck Software).

5.12. Cytopathic effect assays

In a 96-well plate, 1000 pfu of virus was incubated with a drug dilution at a final DMSO concentration of 1.5% for 1 h at 37°C. The volume of this incubation was brought up to 100 μl with Dulbecco's modified Eagle's medium (DMEM; Gibco), 10% fetal bovine serum, 40 mM $MgCl_2$, 3.7% Na_2HCO_3, $1\times$ non-essential amino acids (Gibco), $1\times$ Pen/Strep (Gibco). All drug stocks were made in 100% DMSO. The virus stock was in PBS. After pre-incubating the virus with the compounds, 1×10^4 HeLa cells in a

50 μl volume of the DMEM solution above was added to each well. After 2 days at 37°C in a humidified 5% CO_2 incubator, the plates were fixed with 7% formaldehyde, then stained with 0.1% crystal violet. The MIC was the minimum concentration of compound required to prevent complete lysis of all cells. Six of the most promising candidates were screened by Viropharma for their ability to inhibit a panel of enteroviruses and rhinoviruses using their standard high-throughput screen. Results of these assays were reported as IC_{50} values, which corresponds to the concentration of compound required to protect 50% of the cells in the well plate.

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