Droplet Microarrays

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In Situ Assembly and Screening of Enzyme Inhibitors with Surface-Tension Microarrays**

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Microarray technology has been developed to address the growing number of assayed entities in the modern drug-discovery process.[1] Generally, microarrays are produced by attaching one of the interacting components to the array surface.[1] Subsequent screening of the surface-displayed entities against a liquid sample enables the identification of specifically interacting partners. Unfortunately, such a heterophase assay cannot be applied accurately to the majority of (bio)chemical reactions, which normally proceed in the solution phase. Gosalia and Diamond proposed a simple, yet elegant, solution to this problem by conducting enzymatic reactions in glycerol nanodroplets printed on the surface of a glass slide.[2] This and related techniques have subsequently been used for profiling enzyme activities, inhibitors, and polymeric biomaterials.[3] One limitation of this approach, however, is the evaporation of the droplets, which becomes increasingly important on a nanoliter scale.[4] Thus, the assays must be conducted in solvents of low volatility, such as glycerol and dimethyl sulfoxide (DMSO), which are often not suitable for biochemical reactions. Furthermore, nonimmobilized droplets cannot be used in relatively complex, multistep assays.

We propose herein an approach that, owing to the combination of surface-tension microarrays and piezoelectric dispensing, is optimally suited for both the synthesis and the profiling of enzyme inhibitors (Figure 1). The microarray has a hydrophobic surface patterned with hydrophilic spots to maintain the position of the arrayed droplets.[5,6] This support facilitates the handling of bigger (30–150 nL) droplets and thus alleviates the evaporation problem.

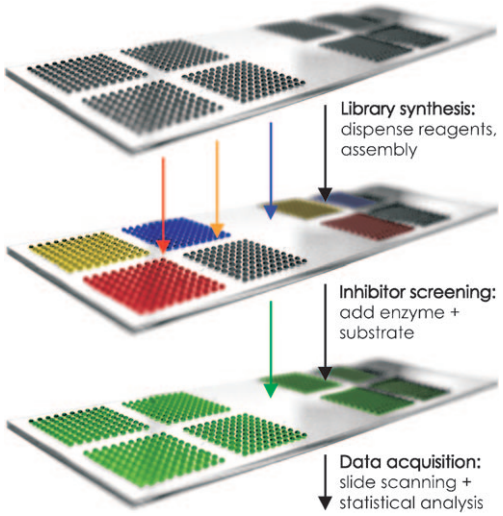

Figure 1. Conceptual summary of the synthesis and screening of enzyme inhibitors on the surface-tension microarray.

We applied this technology to the discovery of new inhibitors of the NS3/4A serine protease of the hepatitis C virus (HCV). HCV is a major cause of chronic hepatitis worldwide, with more than 170 million people infected.[7] The NS3/4A protease is an attractive target for drug development because it plays an essential role in maturation and immune evasion of the virus.[7] We show herein that new NS3/4A inhibitors can be identified readily by using simple assembly chemistry, without any prior structural considerations. Notably, one of the compounds identified had submicromolar potency and could potentially be used as a scaffold in further drug design. Our demonstration of 1) the quantitative analysis of enzyme kinetics on microarrays and 2) the solution-phase organic synthesis and consecutive screening of molecules opens new applications for microarrays in drug discovery.

The glass slides were prepared with 800 hydrophilic spots of 500 μm in diameter. The spots were grouped in eight separated blocks on the hydrophobic surface (see Figures S1 and S2 in the Supporting Information).[5] First, we examined the possibility of synthesizing enzyme inhibitors directly on this support, with the aim of screening them subsequently on the same microarray (Figure 1). We chose to examine the condensation of aldehydes with hydrazides as an efficient and traceless reaction[8,9] compatible with an enzymatic assay.[9,10] 3′,5′-Diformylphenylboronic acid (DFPB) was used as a scaffold for library assembly (Figure 2). Phenylboronic acids

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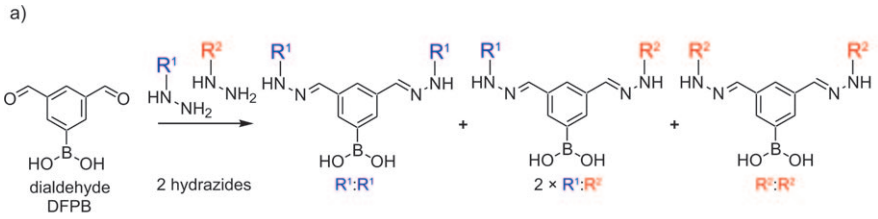
a)



Number of compounds = $\frac{n!}{2!\,(n-2)!}$ (asym.) + n (sym.) = 20100 (n = 200)

b)

Hydrazides	DFPB conversion [%] 24 h, 25°C	10 min, 80 W
18 + 61	57	100
25 + 168	35	100
97 + 171	72	100
106 + 122	62	100
142 + 181	31	100

c)

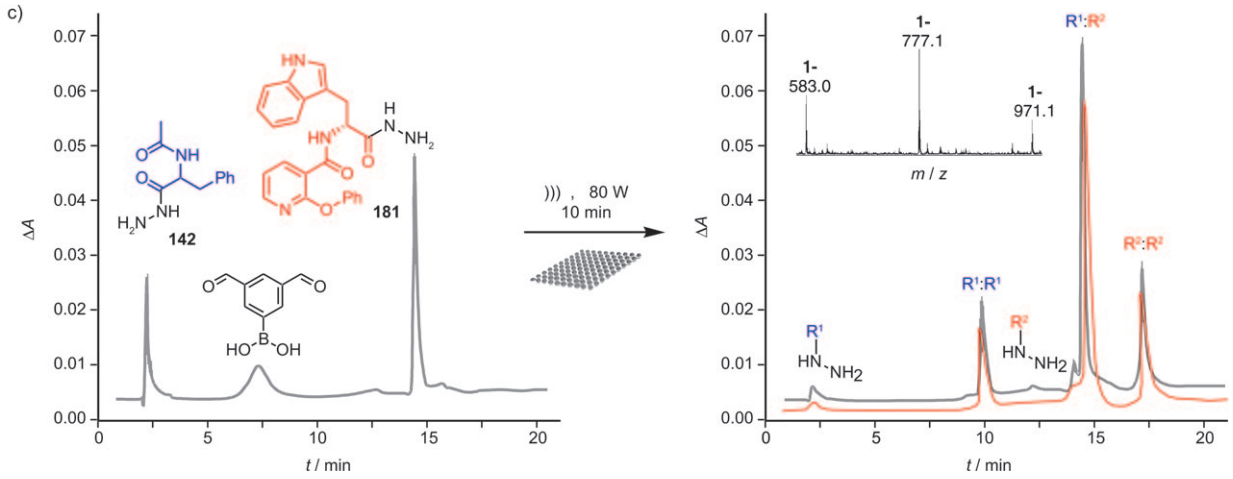


Figure 2. Chemical synthesis on a patterned support. a) General scheme for the synthesis of a dihydrazone library from DFPB and 200 hydrazides. b) Reaction of DFPB with five selected hydrazide couples under two different sets of conditions (hydrazide numbering and structures are given in Figure S3 in the Supporting Information). The conversion of DFPB was measured by HPLC. c) HPLC chromatograms of the reaction of DFPB with a representative pair of hydrazides (**142** and **181**). The initial reaction mixture is shown on the left. The chromatogram on the right shows the formation of two symmetrical dihydrazones, **142**:**142** (t_r = 10.06 min; ESIMS: m/z calculated for $[M-H]^-$: 583.2; found: 583.0) and **181**:**181** (t_r = 17.56 min; ESIMS: m/z calculated for $[M-H]^-$: 971.3; found: 971.1), and one asymmetrical dihydrazone, **142**:**181** (t_r = 15.01 min; ESIMS: m/z calculated for $[M-H]^-$: 777.3; found: 777.1). The inset shows the ESIMS spectrum of the reaction mixture (see Figures S9–S11 in the Supporting Information). The red line is the chromatogram of the reaction mixture when the reaction was carried out in a standard flask.

are known inhibitors of serine proteases.[11] DFPB, in particular, has two aldehyde groups, which could be used in a combinatorial reaction with hydrazides. This approach ensured that all synthesized molecules would have a boronic acid functionality and would thus be biased to inhibit serine proteases. The library was synthesized on the slides by treating DFPB with two hydrazide reagents (Figure 2a).

We generated a set of 200 hydrazides, which enabled the synthesis of 20100 distinct dihydrazones (without taking into account *syn*/*anti* isomerism; Figure 2a; see also Figures S3 and S8 in the Supporting Information). Five hydrazide pairs were examined to validate the synthesis on the patterned support (Figure 2b). Each reaction was conducted on an individual slide in 100 nL DMSO/glycerol (9:1) droplets containing DFPB (2 mM) and the appropriate hydrazides (each 2.1 mM). At various times, the droplets were combined and analyzed by HPLC and ESIMS. We found that microwave irradiation of the slides for 10 min at 80 W resulted in quantitative conversion of DFPB irrespective of the hydrazide used (Figure 2b,c). Notably, the HPLC profile of reaction products on the slides was essentially the same as for synthesis in a reaction flask (Figure 2c). ESIMS analysis confirmed the formation of the corresponding dihydrazones (Figure 2c; see also Figures S9–S11 in the Supporting Information). The acceleration of chemical reactions on planar supports by microwaves has been reported previously and most likely results from heating and concentration effects.[12] Indeed, we observed that microwave treatment led to almost complete evaporation of the arrayed droplets. The reaction thus proceeded quantitatively, irrespective of the hydrazide structure or initial concentration of the droplets.

Because the measurement of reaction kinetics is a necessary prerequisite for the profiling of enzyme activity, we next set up an enzymatic assay on a patterned support. Previous studies of enzyme kinetics with microarrays were limited to heterophase assays with biomolecules attached to the surface.[13] As a result, application of the classical Michaelis–Menten model for enzyme kinetics was complicated.[13] Therefore, we examined whether the conditions of a standard enzymatic assay could be reproduced in droplet arrays. As a test, we performed kinetic and inhibition analysis of the cleavage of the fluorogenic substrate SB1 by recombinant NS3/4A protease. Solutions of the protease and the substrate were mixed on a cooled microarray support, and the

reaction was initiated by transferring the slide to a humidified chamber at 37 °C. In these reactions, the protease (10 nM) and the substrate (0.5–32 μM) were combined in 100 nL droplets. Analysis of the slides showed time- and concentration-dependent increases in droplet fluorescence at 520 nm (Figure 3a; see also Figure S4 in the Supporting Information). Enzyme kinetics were measured for each spot by converting the fluorescence signal into the amount of cleaved substrate. We found that the initial reaction velocities fitted accurately to a Michaelis–Menten curve, in support of an enzyme-catalyzed mechanism (Figure 3b,c). The extracted reaction parameters ($K_m = 13.9$ μM, $k_{cat} = 1.37$ min^{-1}) were very close to those measured in a "macroscopic" fluorimeter cuvette ($K_m = 11.2$ μM, and $k_{cat} = 2.1$ min^{-1}).

To evaluate the performance of the assay, 100 identical enzymatic reactions were analyzed, and the signal-to-noise ratio (S/N) and quality Z' factor were calculated at different time points (Figure 3d). Although the best performance ($Z' = 0.54$) was attained at 4 h, it was accompanied by some inactivation of the enzyme, as revealed by a decrease in the S/N ratio and a significant deviation of the reaction kinetics from the theoretical curve. Therefore, the reaction time was fixed at 2 h for inhibitor-screening assays. This reaction time led to the best S/N ratio of 36.6 and a satisfactory quality factor, $Z' = 0.52$ (Figure 3d).

Next, we examined the inhibition of the NS3/4A protease with the library core compound DFPB (Figure 3e). For this assay, the protease (final concentration: 10 nM) and the substrate (final concentration: 2 μM) were added to the droplets containing DFPB, and the slide was analyzed after 2 h. We found that, like other nonspecific serine protease inhibitors,[14] DFPB had only a moderate effect on NS3/4A,

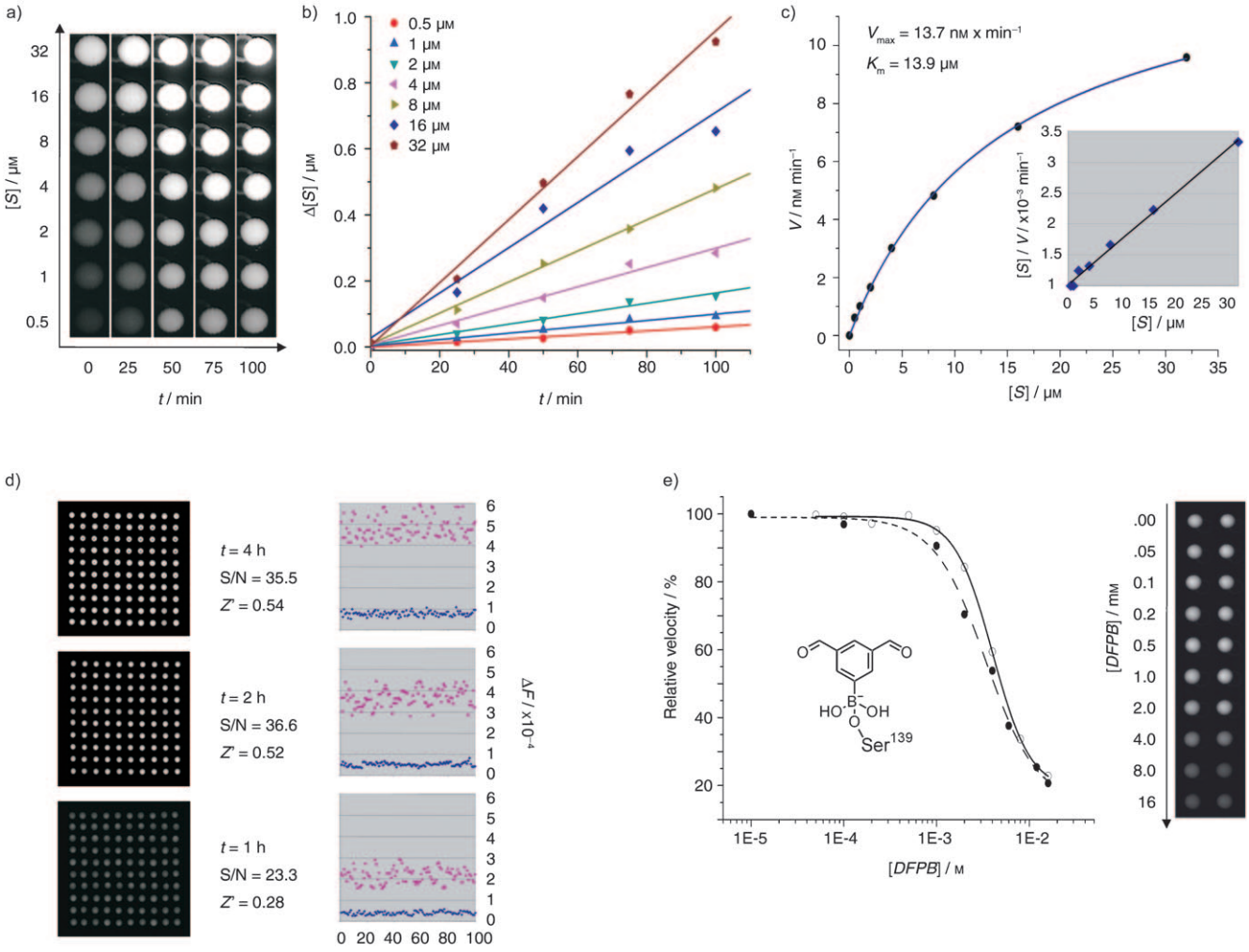


Figure 3. Enzymatic assay on a patterned support. a) Series of scanner images demonstrating the time-dependent cleavage of the fluorogenic substrate SB1 by recombinant NS3/4A protease. b) Quantitative analysis of scanner images in (a). c) Plot of initial velocities, *V*, versus substrate concentration, [S], fitted by a Michaelis–Menten curve. The inset shows the corresponding Hanes–Woolf linear regression. d) Evaluation of assay performance. The images on the left show a time-dependent increase in the droplet fluorescence produced by cleavage of the SB1 substrate (2 μM) with the NS3/4A protease (10 nM). The observed fluorescence intensities (on the right, magenta dots) are shown in comparison with control measurements of spontaneous SB1 hydrolysis (blue dots). The mean values and standard deviations extracted from the data were used to calculate the S/N ratio and the Z' factor. e) Inhibition of the NS3/4A protease by DFPB. The graph compares data from experiments conducted on a microarray (empty circles, solid line) and in a fluorimeter cuvette (filled circles, dashed line). The panel on the right shows the corresponding microarray image.

with a half-maximal inhibitory concentration (IC_{50}) of 4.5 mM (Figure 3e). A similar IC_{50} value of 3.8 mM was obtained in parallel in a fluorimeter cuvette. Taken together, these results demonstrate the feasibility of the microarray assay for the accurate analysis of an enzymatic reaction.

Having set up both the chemical synthesis and the protease assay in a microarray format, we proceeded with inhibitor screening by combining these two approaches on the same support. The library of inhibitors was synthesized by cross-reaction of the hydrazides and DFPB on the glass slide. The resulting acyl hydrazones were diluted with DMSO (10 nL), and the final volume was increased to 100 nL with solutions of the protease (final concentration: 10 nM) and the substrate (final concentration: 2 μM). Since the resulting

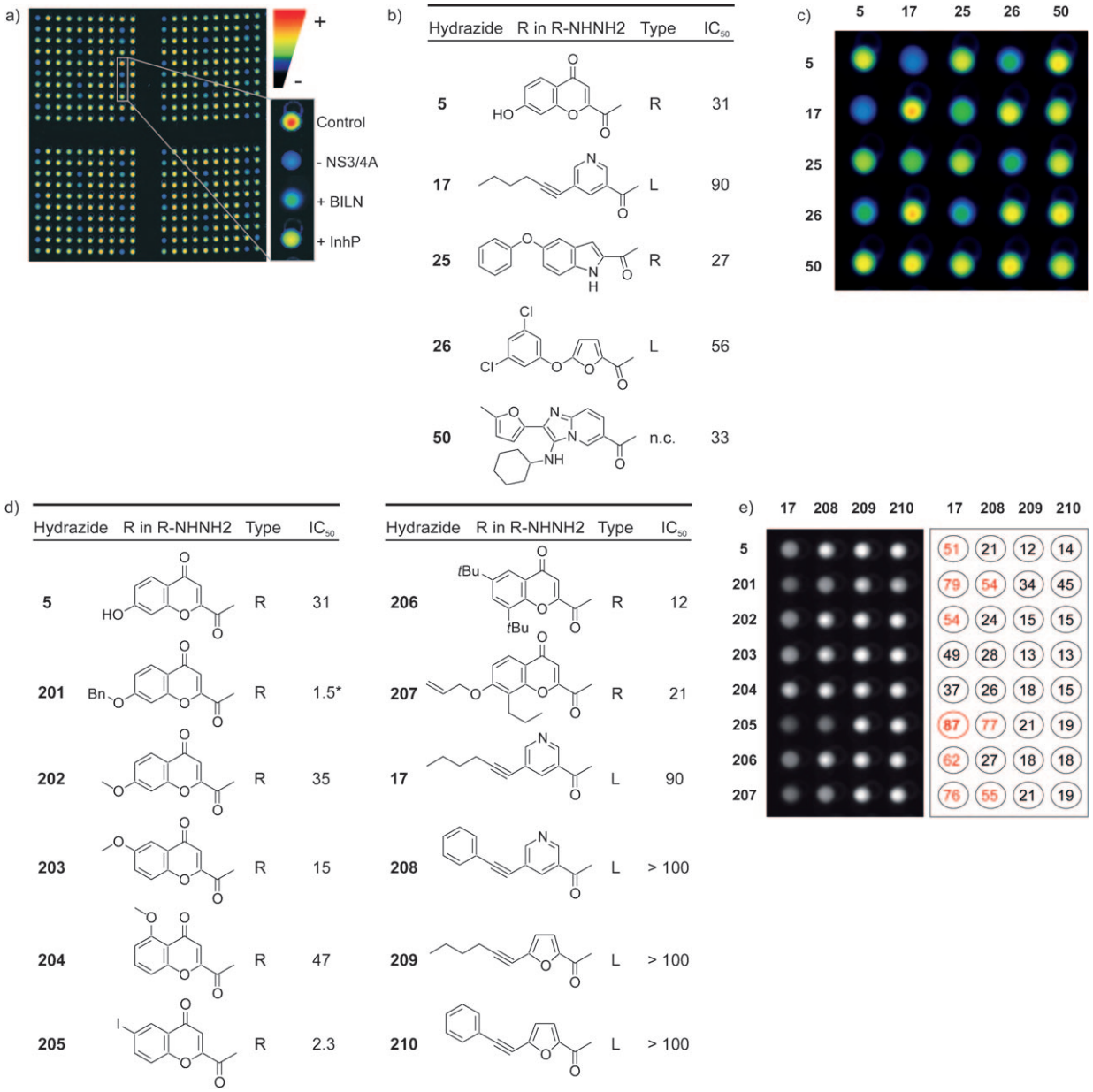


Figure 4. Inhibitor screening on a patterned support. a) Representative fluorescence scanner image of a microarray. The results of a series of control reactions are shown on the lower right: reaction without an inhibitor (control), reaction without the protease (−NS3/4A), reaction with BILN2061 (BILN, IC_{50} = 2 nM), reaction with a peptidic inhibitor (InhP, IC_{50} = 2 μM). b) Structures of the hydrazides that yielded the most potent NS3/4A inhibitors upon condensation with DFPB. All precursor hydrazides were tentatively classified as right (R) or left (L) according to their cooperative inhibitory effect (Figure 4c), except **50**, which could not be classified unambiguously (n.c.). The IC_{50} values (μM) describe the inhibition of the protease with the corresponding symmetrical dihydrazones (see Figure S12 in the Supporting Information). c) Scanner image demonstrating the cooperative inhibitory effect that results from the cross-reaction of R and L hydrazides with DFPB (final concentration: 27 μM). d) Structural analogues of the R hydrazide **5** and the L hydrazide **17**. The IC_{50} values (μM) are for the corresponding symmetrical dihydrazones (see Figure S12 in the Supporting Information). Bn = benzyl. The * denotes that **201** precipitated at a concentration above 3 μM. e) Structure–activity relationship of the precursor hydrazides for the inhibition of the NS3/4A protease. The image on the left shows the inhibitory effect of hydrazones synthesized by the cross-reaction of R and L hydrazides with DFPB (final concentration: 3 μM). The panel on the right shows the percentage of NS3/4A inhibition for a given microarray position; inhibition above 50% is highlighted in red.

reaction solution contained 10% DMSO, we checked the effect of this solvent on the reaction kinetics. About a 21% decline in the SB1-cleavage rate was observed, which resulted in some decrease in the dynamic range (S/N = 28) and quality ($Z' = 0.38$) of the assay. The inhibitors were synthesized to provide a total boronic acid concentration of 27 μM per assay droplet, then resynthesized and rescreened at 12 μM, and finally at 3 μM. In each screen, the effect of the synthesized acyl hydrazones was compared with a set of control reactions (Figure 4a; see also Figure S5 in the Supporting Information).

After multiple screens, the five hydrazides that showed the highest inhibitory effect were selected (Figure 4b). Symmetrical dihydrazones synthesized from these hydrazides were much more potent inhibitors of the NS3/4A protease than DFPB, with IC_{50} values two orders of magnitude lower (Figure 4b; see also Figure S12 in the Supporting Information). Interestingly, analysis of the microarray data revealed that some cross-reactions, such as **17** + **5** + DFPB and **26** + **5** + DFPB, resulted in more pronounced inhibition of the NS3/4A protease than the corresponding reactions with a single hydrazide (**5** + **5** + DFPB, **17** + **17** + DFPB, **26** + **26** + DFPB; Figure 4c). This cooperative effect could be explained by the formation of mixed asymmetrical dihydrazones with significantly higher inhibitor potency than that of the symmetrical dihydrazones. We reasoned that two acyl hydrazone moieties, tentatively defined as right (R) and left (L), might interact cooperatively with distinct parts of the protease active site (see Figure S6 in the Supporting Information).

The most potent couple identified in this screen was composed of the chromone hydrazide **5** (R) and the substituted nicotinic acid hydrazide **17** (L). To further ameliorate inhibitor potency, 10 structural analogues of these compounds were produced from readily available commercial precursors (Figure 4d). A panel of DFPB-based inhibitors was then synthesized by cross-reaction of the R and L hydrazides on a microarray. At a concentration of 3 μM, some pairs inhibited NS3/4A activity more efficiently than the **17** + **5** couple (Figure 4e). Quantitative structure–activity-relationship analysis of microarray images revealed **205** as the most potent R component, followed by **201** and **207** (Figure 4e). Compounds **201**:**201** and **205**:**205** were also the most potent inhibitors among symmetrical dihydrazones (Figure 4d).

Among the L components, the initial hit **17** was the most potent. The compound **17**:**205** was synthesized in milligram quantity and purified by HPLC. When tested with recombinant NS3/4A protease, it showed an inhibition constant (IC_{50}) of 150 nM, which is about 3×10^4 times lower than the IC_{50} value of DFPB (see Figure S13 in the Supporting Information). A small inhibitory effect on virus growth in human hepatoma cells transformed with the HCV-1b replicon was also observed (see Figure S7 in the Supporting Information).[15] The compound **17**:**205** also inhibited chymotrypsin with an IC_{50} value of 12.9 μM, which is 86 times higher than the IC_{50} value observed for the NS3/4A protease. No inhibition of cathepsin B was observed, as a further demonstration of the specificity of the inhibitor. This specificity can partially be explained by the three-dimensional model of the **17**:**205** interaction, which shows the chromone moiety in contact with the protease S3 residues Ala157 and Cys159 and a nicotinic moiety found near Lys136 in the S2′ specificity pocket (see Figure S6 in the Supporting Information).

In conclusion, this study has established the concept of surface-tension microarrays as a tool for both the solution-phase chemical synthesis of small molecules and the high-throughput quantitative analysis of enzyme kinetics. We have demonstrated that organic synthesis and the screening of enzyme inhibitors can be combined on the same microarray. In this approach, a chemical library is assembled in situ from a relatively small set of building blocks; thus, the screening of thousands of fully elaborated compounds is avoided. With the right choice of a common building block, the synthesized library can be biased to inhibit a specific class of enzymes. The method relies on highly selective and efficient reactions used in such combinatorial approaches as the creation of dynamic combinatorial libraries[9] and click chemistry.[16] The advantage of the microarray is that the division of the complex combinatorial library into hundreds of separated reactions simplifies the analysis of screening data significantly and alleviates constraints on library size. The use of a piezoelectric dispenser provides the additional advantage of addressing the composition of each droplet independently. Hence, surface-tension microarrays represent a real miniaturization of a more general drug-discovery approach, in which reactions on microtiter plates are used for in situ synthesis and the screening of enzyme inhibitors.[17] However, reagent consumption is approximately 1000 times lower on microarrays than on microtiter plates. Because neither the reacting component nor the reaction support requires specific modification, this approach can be applied to any (bio)chemical assay. Therefore, although we focused our efforts on a protease assay, other important drug targets, such as kinases, phosphatases, and receptor proteins, could also be addressed.

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