COMMUNICATION

Picomole-scale synthesis and screening of macrocyclic compound libraries by acoustic liquid transfer

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Abstract: Macrocyclic compounds are an attractive class of therapeutic ligands against challenging targets such as protein-protein interactions. However, the development of macrocycles as drugs is hindered by the lack of large combinatorial macrocyclic libraries, which are cumbersome, expensive, and time consuming to make, screen, and deconvolute. Here, we established a strategy for synthesizing and screening combinatorial libraries on a picomolar scale using acoustic droplet ejection to combine building blocks at nanoliter volumes, which reduced reaction volumes, reagent consumption, and synthesis time. As a proof-of-concept, we assembled a 2,700-member target-focused macrocyclic library that we could subsequently assay in the same microtiter synthesis plates, saving the need for additional transfers and deconvolution schemes. We screened the library against the MDM2-p53 protein-protein interaction and generated micromolar and sub-micromolar inhibitors. Our work synthesizing combinatorial macrocycle libraries at the picomole-scale using acoustic liquid transfer provides a general strategy towards macrocycle ligand development.

Macrocyclic compounds are of great interest to the pharmaceutical industry due to their ability to bind to challenging targets such as proteins with flat surfaces or protein-protein interactions, for which it has been difficult or impossible to generate ligands based on classical small molecules. Of particular interest are membrane permeable macrocyclic compounds that can reach intracellular targets, which requires that they are small (< 1 kDa) and have a limited polar surface area (< 200 Å^2).[1] However, the development of macrocyclic ligands within these parameters is hindered by the small size of commercially available libraries (typically around 10,000 molecules or smaller)[2–4] and the lack of efficient synthetic methodologies to generate target-focused libraries. Impressive progress to access larger macrocyclic compound libraries was made in recent years using DNA-encoding technologies,[5–8] but the synthesis of combinatorial macrocycle libraries based on split and pool methods and subsequent deconvolution can be challenging due to accumulation of side products in the many sequential coupling steps needed in macrocycle compounds synthesis. To access combinatorial libraries comprising ten-thousands of macrocycles while limiting the synthesis and deconvolution complexity, our laboratory has recently established an approach for library synthesis in microwell plates by the sequential "adding and reacting" of building blocks.[9] From an 8,988-member macrocycle library, we identified nanomolar inhibitors of protease targets. While this approach showed great promise for the development of macrocyclic ligands, we were limited by the high costs of pipetting tips used for liquid transfer and the mg-scale amounts of peptide needed as building blocks for synthesis.

Combinatorial library synthesis can potentially be miniaturized by performing reactions in nanoliter (nL) volumes. Previously, Dreher, Cernak, and co-workers used mosquito nL dispensing, an approach in which droplets are transferred by thin pins, to combine reagents and test many conditions for complex small-molecule synthesis.[10] This high-throughput nanomole-scale approach was subsequently used with an affinity-selection mass spectrometry bioassay to test 3,114 reaction conditions for 345 compounds.[11] Dömling and co-workers used acoustic droplet ejection (ADE) technology for the transfer of reagents in nL volumes and pioneered both, the scouting of chemical reactions and the combinatorial synthesis of drug-like scaffolds by ADE[12,13], and they recently synthesized and screened a library of several hundred small molecules at a 500 nmol scale.[14] With ADE, acoustic sound is used for contact-free liquid transfer in nL volumes from wells of a source plate to wells of an inverted destination plate positioned above.[15] The ADE technology has been broadly applied for the high-throughput screening of small-molecule libraries in the pharmaceutical industry, in which small molecules are transferred to assay reagents in microtiter plates.[15] Zhang et al. have combined ADE with mass spectrometry for efficient reaction analysis.[16] To our knowledge, ADE has not been used to synthesize combinatorial compound libraries at a picomole scale for subsequent sampling of target engagement in high-throughput screens.

Herein, we applied acoustic dispensing to synthesize large combinatorial macrocyclic compound libraries by step-wise transfer of building blocks to 384-well microtiter plates, as illustrated in Figure 1a. In brief, *m* building blocks (in blue) were mixed with *n* building blocks (in yellow). Subsequently, *o* building blocks (in green) were added to cyclize the linear products to yield $m \times n \times o$ macrocyclic products, with each combination in a discrete well. We envisioned that the efficiency of coupling and cyclization reactions would yield macrocyclic compounds as the main product that can be screened without purification, simply by adding assay reagents to the synthesis plate.

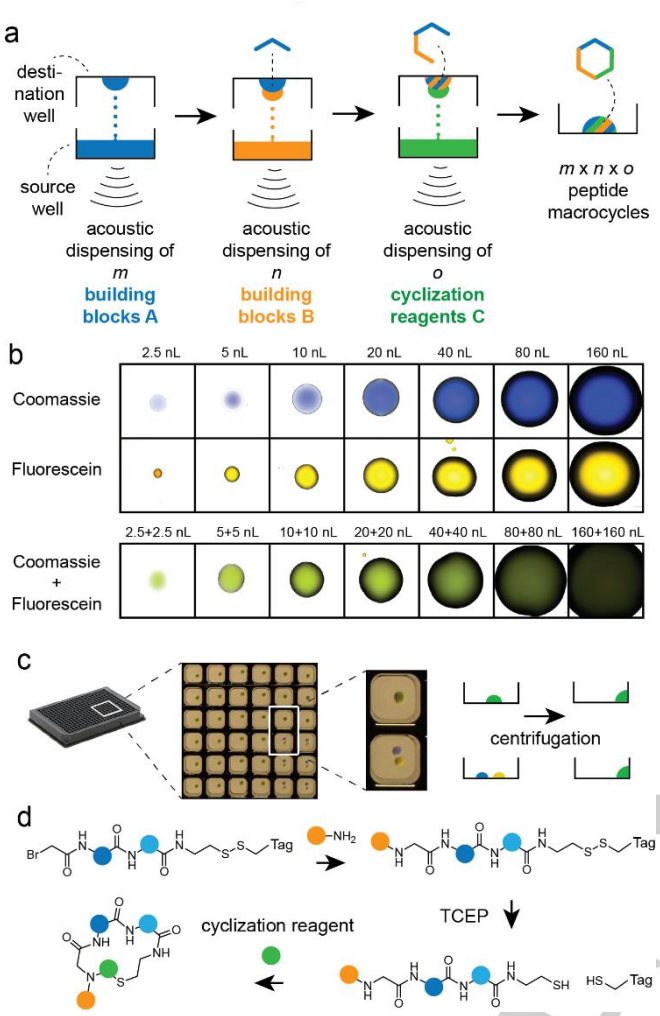

Figure 1. Combinatorial macrocyclic compound synthesis by acoustic droplet ejection (a) Building blocks are transferred step-wise from source to destination wells in microtiter plates. Acoustic waves (indicated schematically) applied to the lower source wells transfer 2.5 nL droplets to the inverted destination well above. The three different building blocks are indicated by color. The product macrocycle is contained in the blue-yellow-green droplet. (b) Microscopy images (10× amplification) of DMSO containing Coomassie (blue), fluorescein (yellow) or both (green). Solutions were transferred as 2.5-nL pulses to generate droplets of 2.5 to 160 nL. (c) Droplet merging in a 384-well microtiter plate. Two enlarged wells demonstrated merged (upper) or unmerged (lower) droplets. Droplets in all wells are merged by centrifugation of the plate. (d) Macrocycles are synthesized using a three-component strategy. Colored spheres represent varied positions in the combinatorial library. The cysteine thiol group is here protected by a disulphide-bridged short peptide (Tag).

We first assessed the efficiency of reagent mixing in nL-sized droplets as transferred by acoustic sound using colored liquids and a standard acoustic transfer device (ECHO 650 from Labcyte/Beckman Coulter) (Figure 1b). The transfer of water was problematic for volumes smaller than 40 nL due to rapid air-drying (not shown). Transfers of reagents in DMSO or in DMSO-H_2O mixtures were efficient and prevented drying, even in droplets as small as 2.5 nL. We transferred reagents of two different colors, blue Coomassie and yellow fluorescein, in 2.5-, 5-, 10-, 20-, 40-, 80- or 160-nL volumes and found that the merged droplets immediately turned green, which indicated efficient mixing of the compounds in the droplets and solvent (Figure 1b).

We endeavored to use rather low reaction volumes in the double-digit nL range as this would limit the number of acoustic pulses (liquids are transferred in pulses of 2.5-nL volumes) and generate high reagent concentrations for efficient building-block coupling. However, we were concerned that at small volumes, the droplets of two reagents might occasionally not meet. The transfer of 40 nL volumes of two compounds to all wells of a 384-well plate revealed that droplets of this size merge in almost all but not all cases (Figure 1c and Figure S1). Further optimization of the transfer protocols helped somewhat, but in the end, we solved this by introducing a centrifugation step that ensured robust droplet merging (Figure 1c and Figure S2).

We tested the acoustic dispensing strategy using a recently developed macrocycle synthesis approach (Figure 1d) in which *m* N_α-bromoacetyl-activated linear peptides were reacted with *n* primary amines, followed by a cyclization with *o* linkers to yield *m* × *n* × *o* macrocycles.[17] We had previously used this approach to affinity mature a macrocyclic thrombin inhibitor (P2, K_i = 42 nM) through combinatorial synthesis of thousands of variants of it. However, we had not applied the strategy to the synthesis or screening of random macrocyclic compound libraries, nor to any target other than thrombin. We first tested whether the three consecutive reactions, previously performed in larger reaction volumes of 20 µL (alkylation), 96 µL (reduction), and 100 µL (cyclization), were possible in 80 nL to 4 µL volumes, which were up to 250-fold smaller volumes, as shown in Figure 2a. We performed the three consecutive reactions with the three building blocks shown in Figure 2a: i) a BrAc-activated model peptide (protected at the thiol group by a disulfide conjugated short peptide tag that helped also for its purification; Figure S3), ii) an example primary amine (2-furanmethanamine), and iii) cyclization reagents such as divinyl sulfone (**1**) (Figure 2a). The alkylation and disulfide-reduction reactions succeeded in the small volumes with over 95% yields (Figure 2b; desired products in red). The subsequent cyclization reactions with the seven tested linker reagents (Figure 2c) had yields ranging from more than 60% (reagents **1**-**3**, 61, 66, and 69%), to low or almost no product (reagents **4**-**7**, 2, 0.2, 26, 0.8%; Figure S4). We considered the yields of reagents **1**-**3** as sufficiently good for library synthesis and screens, and thus proceeded with them.

We next tested whether the macrocycle libraries synthesized by acoustic dispensing were adequate for identifying ligands against a target of interest. As a proof-of-concept, we chose the oncology-driving protein-protein interaction between p53 and MDM2 as a target. Overexpression of MDM2 inhibits the activity of the tumor repressor p53, and MDM2 binders that inhibit the MDM2-p53 interaction have been shown to suppress tumor growth.[18] To increase the chances of finding hits, we tailored the library design to include the amino acid 6-chloro-tryptophan ($Trp^{6\text{-}Cl}$) in each macrocycle. $Trp^{6\text{-}Cl}$ is a building block that forms the key interaction with MDM2 in short linear and stapled peptides.[19] To ensure it was in every macrocycle, we designed the library with the scheme shown in Figure 3, indicating how $Trp^{6\text{-}Cl}$ was incorporated in either the C-terminal (Library 1) or the N-terminal region (Library 2). To make the complete macrocycle, $Trp^{6\text{-}Cl}$ was accompanied by one of 10 randomly chosen amino acids that differ strongly in their backbones (i.e. α-, β-, γ- and ε-amino acids; **8**-**17**; blue in Figure 3), one of 45 amine building blocks (**18**-**62**, yellow in Figure 3), and one of three cyclization linkers (**1**-**3**, shown in Figure 2c).

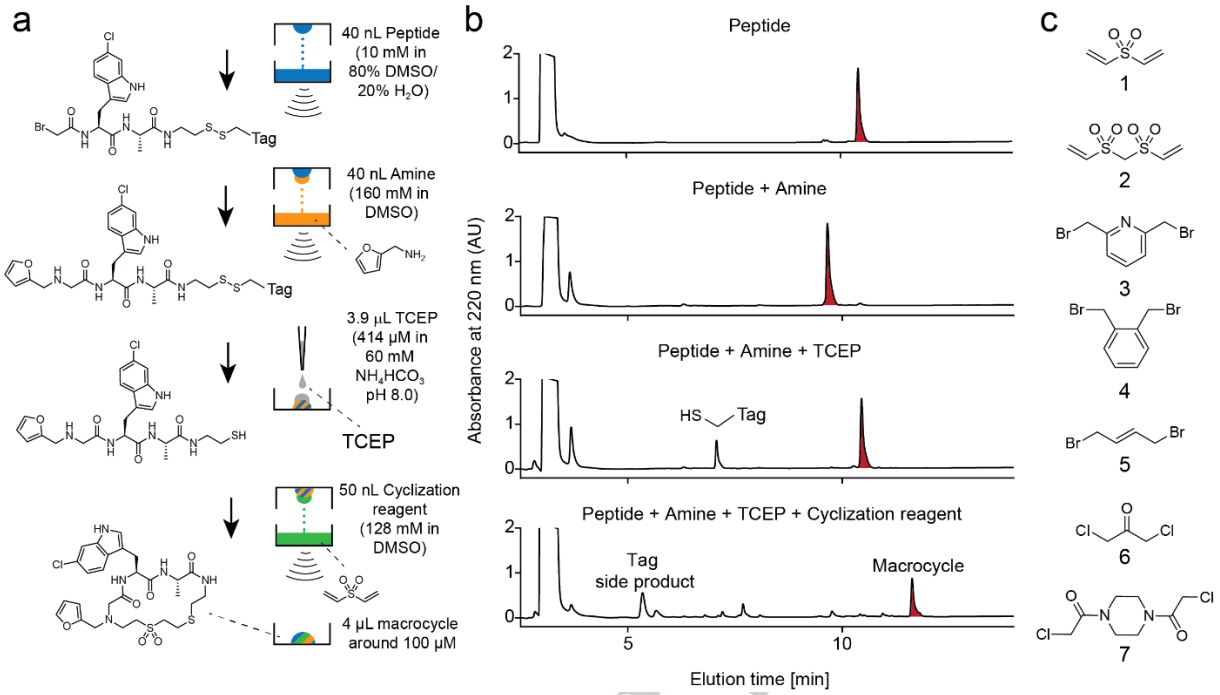

Figure 2. Macrocycle synthesis by acoustic liquid transfer. (a) Representative chemical structures of reagents, intermediates, and the final macrocyclic product. The peptide (blue), amine (yellow), and cyclization reagent (green) are transferred by acoustic sound. TCEP (grey) is transferred by a bulk pipette tip dispenser. (b) HPLC chromatograms of reactions analyzed at various steps during the synthesis. Peaks of desired products are highlighted in red. (c) Variations of bis-electrophile cyclization reagents used in macrocycle synthesis.

To generate the peptide building blocks in the library, we synthesized and purified 10 bromoacetamide-functionalized peptides of the format BrAc-Trp$^{6\text{-Cl}}$-Xaa-Cys*-S-S-Tag (Library 1; Xaa = any of 10 structurally diverse amino acids; Cys* = cysteamine) and 10 of the format BrAc-Xaa-Trp$^{6\text{-Cl}}$-Cys*-S-S-Tag (Library 2), all with a short, disulfide-linked peptide tag that protected the thiol group and facilitated the purification (Tag = mercaptopropionic acid-Ser-Gly-Arg-Tyr). The 2,700 macrocycles were assembled robotically in 384-well plates by acoustic dispensing using the reagents, volumes, concentrations, and reaction conditions as indicated in Figure 2a. We obtained the final macrocyclic elements in volumes of 4 µL and at 100 µM, the latter concentration hypothetically assuming quantitative conversion of linear peptides to macrocycles.

We screened the library for MDM2-p53 inhibitors by dispensing 11 µL of human MDM2 solution (as GST fusion; Figure S5; 1 µM in assay) and a fluorescent-labeled reporter peptide[20] (K_d = 0.5 µM; Figure S6; 50 nM in assay) to the 384-well synthesis plates containing the 2,700 macrocycles (26.7 µM in assay). Macrocycles that bound MDM2 displaced the reporter peptide and caused a decrease in fluorescence polarization, as shown in Figure 4a. The extent of reporter peptide displacement by the macrocycles is shown for Library 1 in the heat map in Figure 4b (see Library 2 in Figure S7).

Several combinations of BrAc-peptide, amine, and cyclization reagent induced strong displacement of the reporter peptide, as indicated by the dark-red to black colors in the heat map. Control reactions in which no cyclization reagent was added showed weaker signals, which suggests that macrocyclization is necessary for strong binding activity.

To determine the reproducibility of the synthesis and screening assays and to verify that the activities observed during the screen were from the macrocyclic products, we studied macrocycles 1, 2 and 3 in greater depth (Figure 4b and Figure 5a). We repeated twice the synthesis and activity assay with the crude products which yielded the same results (Figure 5b). In addition, we repeated the reactions of all the 17 hits as well as of 16 randomly chosen reactions in triplicate, and found activities comparable to those in the screen (Figure S7d).

Separation of the reaction products by HPLC for an example reaction (macrocycle 1) showed that the desired macrocycle represented the main product (Figure 5c; fraction 9 highlighted in red), and that the fraction containing the macrocycle was active (Figure 5d and Figure S8). This result showed that the activity observed in the screen for this reaction resulted from the macrocycle and not a side product. A comparison of the activity of HPLC-purified macrocycles 1-3 showed that macrocycles 1 (IC_{50} = 2.7 ± 0.3 µM) and 2 (IC_{50} = 4.3 ± 1.5 µM) were more active than 3 (IC_{50} = 25 ± 2 µM), which was in line with the screening result assaying non-purified products (Figure 5a and 5e). From these results, we concluded that acoustic liquid transfer and combinatorial assembly of building blocks in nL volumes is suitable for the synthesis and function-based screening of combinatorial libraries.

As further validation, we analyzed three additional hits from Library 1 (macrocycles 4, 5, and 6) and 11 hits from Library 2 (Figure S9, S10, and S11). In the reactions for macrocycles 4-6, we found a linear side product that was the most active species and we thus did not further analyze these three macrocycles. The observation that a side product can occasionally be more active

than the desired product was previously found in libraries that we synthesized by pipetting,[9] and underscored the importance of hit verification using purified products.

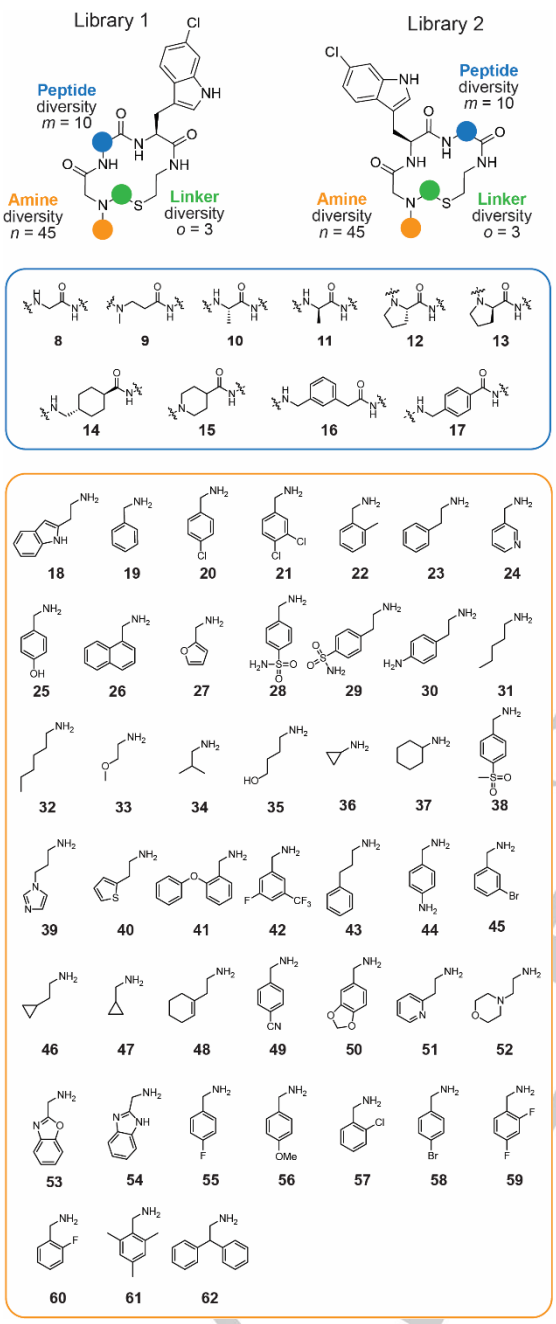

Figure 3. Macrocycle library design and building blocks. All macrocycles contain the amino acid $Trp^{6\text{-}Cl}$ in one of the two possible positions (C-terminal side: Library 1, N-terminal side: Library 2), a variable amino acid 8-17 (blue), an amine 18-62 (yellow), and a linker 1-3 (linkers are shown in Figure 2c).

From Library 2, all 11 hits studied were active macrocycles. Three of them were more potent than the two high-performing hits of Library 1, with the greatest inhibition achieved by macrocycle 9 (IC_{50} = 0.6 ± 0.2 μM), as shown in Table 1. We finally tested the binding of the best two macrocycles by surface plasmon resonance (SPR), which confirmed target-specific binding and dissociation constants in the low micromolar range (macrocycle 8: 11 μM, macrocycle 9: 5.5 μM) (Figure S12).

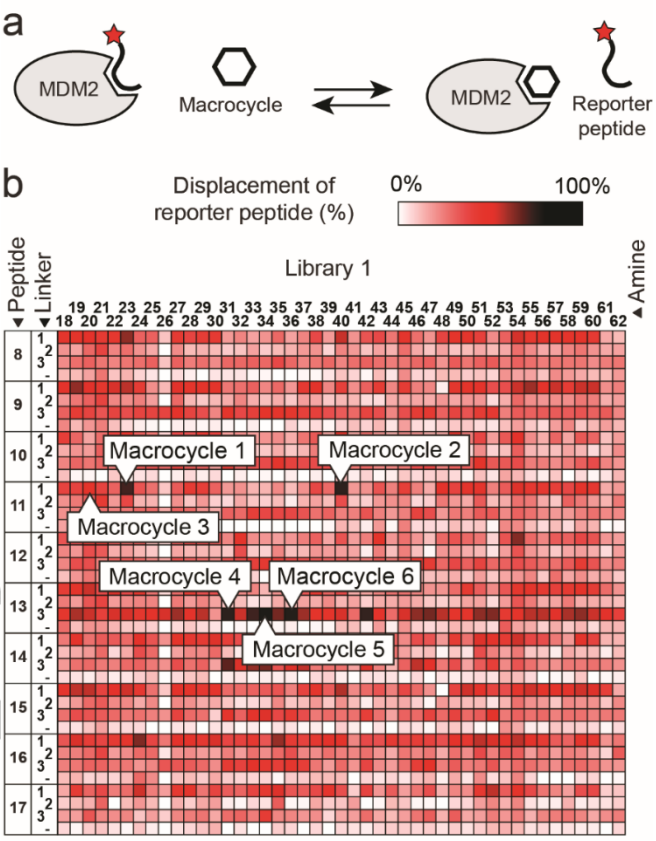

Figure 4. Screen for MDM2-p53 PPI inhibitors. (a) General schematic of the fluorescence polarization competition binding assay. (b) Binding of the 1,350 macrocycles of Library 1 to MDM2. The binding was assessed by displacement of a fluorescein-labeled MDM2-bound reporter peptide using fluorescence polarization. Darker colors indicate higher binding affinity. The building blocks used for library synthesis (shown in Figures 2c and 3) are indicated by number. Control reactions (linear peptides with no linker) are indicated by "-". Lead macrocycles (1 to 3) that were re-synthesized, purified, and characterized are indicated on the heat map. The screening result of library 2 is shown in Figure S7.

In summary, we have developed an efficient approach for the synthesis of combinatorial macrocycle libraries at a picomole scale by sequential transfer of three building blocks in nL-droplets using acoustic liquid dispensing. While ADE has previously been used for combinatorial reaction scouting and screening at a nanomole scale, our work is a *de novo* example of combinatorial library generation and screening at a picomole scale, illustrated with macrocycles. For classical HTS approaches, library compounds are often produced in milligram quantities, corresponding to a micromole scale, which is a million fold more than in the herein presented picomole scale synthesis and screening approach. We have demonstrated the efficiency of combinatorial library synthesis with ADE and identified low- to sub-micromolar inhibitors of the MDM2-p53 oncology target with target-focused macrocycles that contain $Trp^{6\text{-}Cl}$ to drive affinity for MDM2. While we synthesized and screened in 384-well plates, the ADE device used herein is also compatible with 3,072-well plates. In principle, macrocycle synthesis and screening can be conducted at even higher throughput and smaller scale, which will

further reduce reagent use, material consumption, time per synthesis and increase library size and diversity. There are additional advantages of the ADE-based library approach, such as the omission of pipetting tips, which reduces the cost by around 0.3 cents per macrocycle and thus a large sum for libraries comprising thousands of macrocycles. Additionally, the innovative use of microwell plates for overall screening and sampling eliminates the need for large library storage and only requires preservation of the building blocks, which use a much smaller storage space. Taken together, this work provides a general strategy for the rapid combinatorial synthesis of libraries at the picomole scale to generate macrocyclic ligands with potential therapeutic applications.

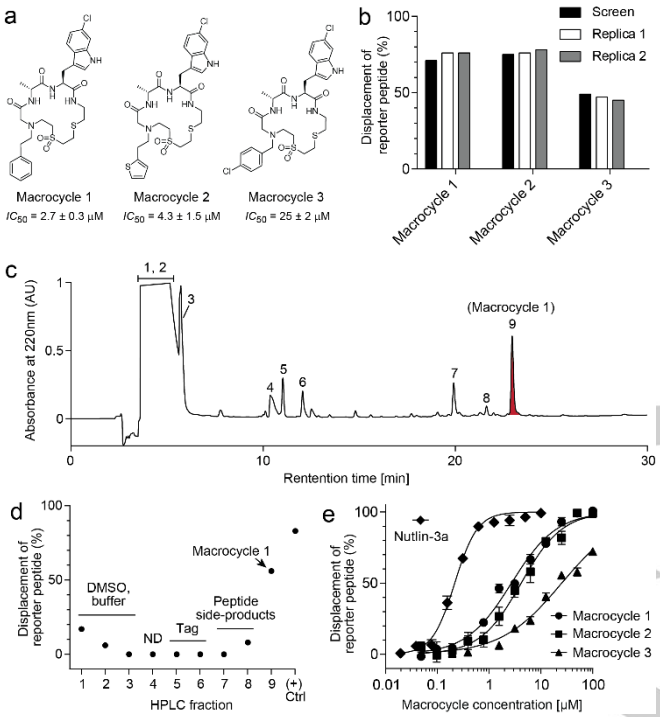

Figure 5. Characterization of hits from screen with Library 1. (a) Structures and IC_{50}s of macrocycles 1-3. Mean values and standard deviations (SDs) of three independent measurements are shown. (b) Macrocycles 1-3 were synthesized and assayed for MDM2 binding as under the screening conditions with little deviation in performance. (c) HPLC-chromatographic separation of the library well containing macrocycle 1. Collected peaks are indicated by numbers. Macrocycle 1 is the product in peak 9 (red). (d) Activity of products in HPLC fractions determined by the MDM2 fluorescence polarization competition assay. Non-labeled reporter peptide was used as a positive control. (e) MDM2 binding of purified macrocycles 1-3 and a control MDM2 binder (nutlin 3a) determined by fluorescence polarization competition assay.

Acknowledgements

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Keywords: macrocycles libraries• combinatorial synthesis • acoustic droplet ejection • picomole scale • protein-protein interaction

Table 1. Binding affinities of purified macrocycles for MDM2.

	Macrocycle	Linker	IC_{50} (µM)
Library 1	1	1	2.7 ± 0.3
	2	1	4.3 ± 1.5
	3	1	25.0 ± 2
	4	3	N.D.
	5	3	N.D.
	6	3	N.D.
Library 2	7	1	14.2 ± 1.9
	8	1	1.3 ± 0.8
	9	1	0.6 ± 0.2
	10	1	2.4 ± 0.9
	11	1	6.9 ± 1.8
	12	2	7.5 ± 0.6
	13	2	8.2 ± 1.1
	14	2	13.1 ± 2.7
	15	2	7.6 ± 2.0
	16	2	3.7 ± 0.5
	17	2	9.8 ± 4.2

IC_{50}s were determined by the MDM2 fluorescence polarization competition assay. Mean values and SDs of three independent measurements are indicated. N.D.: IC_{50}s were not determined as the most active species in the reactions of the screen were side products based on linear peptides.

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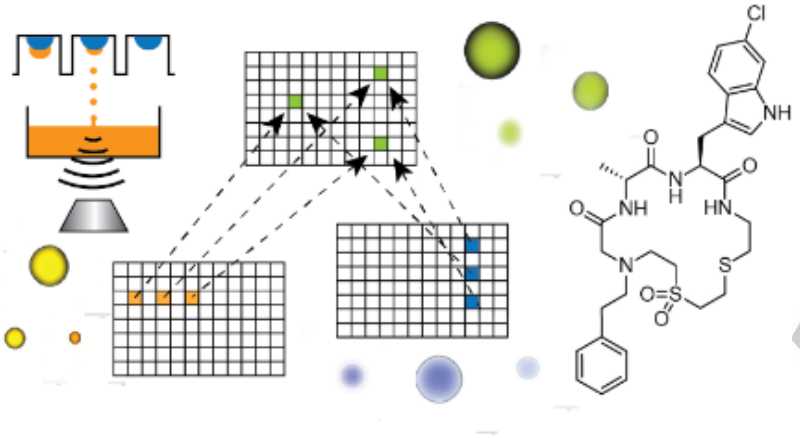

A new approach for synthesizing and screening large libraries of macrocyclic compounds at a picomole scale was developed. The macrocycles were synthesized by combinatorially transferring reagents in 2.5 nL droplets to microwell plates using acoustic waves. In a proof-of-concept study, a library of 2,700 macrocyclic compounds was synthesized and potent inhibitors of the protein-protein interaction p53/MDM2 were identified.

SUPPLEMENTARY INFORMATION

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MATERIALS AND METHODS

Merging reagents by acoustic liquid transfer

For testing the mixing of reagents and merging of droplets, liquids were transferred using an ECHO 550 or ECHO 650 device from Labcyte. All liquids were transferred in droplets of 2.5 nL using settings for DMSO or aqueous solvent transfer. For testing the mixing of transferred compounds, liquids saturated in fluorescein or Coomassie Brilliant Blue in DMSO were transferred from a 384-well ECHO low dead volume source plate (Labcyte 384LDV, Cat. # LP-0200) to a 96-well destination plate (Greiner Bio-One, 96 well, clear, flat bottom, polystyrene microplate). The droplets were visualized and imaged with a microscope (10×, EVOS FL Auto imaging, ThermoFisher Scientific).

For testing the transfer and merging of 40 nL droplets in microtiter plates, saturated solutions of fluorescein (80% [v/v] DMSO/ 20% [v/v] mQ water) and Coomassie Brilliant Blue (DMSO) were transferred from a 384-well ECHO low dead volume source plate to a 384-well low volume destination plate (ThermoFischer NUNC™ 384 shallow well std height plate, non-sterile, black, Cat. # 264705) using settings for DMSO transfer. The droplets were transferred in the following order: first 40 nL of fluorescein followed by 40 nL Coomassie Brilliant Blue. For enforcing merging of non-merged droplets, the plate was centrifuged at 2,000 × g (force measured at 11.5 cm from the centrifuge axis) corresponding to around 4,000 rpm on a Thermo Scientific Multifuge 3L-R centrifuge and a Sorvall swing-out rotor. The droplets were imaged using a digital camera (Olympus OMD EM-5, 16 megapixel, camera with an Olympus M. Zuiko Premium 60/2.8 ED Macro lens and settings of 60 mm, F/13, 1/6 s, ISO 200).

Synthesis of thiol-protected and N^{α}-bromoacetyl-functionalized peptides

Peptides of the format BrAc-Xaa-Yaa-Cys*-S-S-Tag (Xaa and Yaa are the variable amino acids, Cys* = cysteamine. Tag = mercaptopropionic acid-Ser-Gly-Arg-Tyr) were synthesized on solid phase using Rink amide MBHA resin (50 μmol scale, loading of 0.3 mmol/g) in 5 mL syringe reactors using an automated peptide synthesizer (Intavis, MultiPep RSi). In all washing steps, 2 mL of DMF was used and the resin shaken for 1 min at 400 rpm.

The resin was swollen by adding three times 2.4 mL of DMF for 10 min. The amino acids (4.2-fold equiv., 0.22 M final conc.), the coupling agent HATU (3.9 equiv., 0.21 M final conc.) and the base NMM (10 equiv., 0.53 M final conc.), all in DMF, were mixed, 5 μL of NMP added to improve the solubility, incubated for one minute, transferred to the solid phase, and shaken for 45 min at 400 rpm. All Fmoc amino acids and the Trt-protected mercaptopropionic acid were coupled twice. The resin was washed 7 times with 2 mL DMF. Fmoc protecting groups were removed by performing two cycles of 0.8 mL 20% (v/v) piperidine in DMF for 5 min. After conjugation of mercaptopropionic acid, the resin was washed three times with 600 μL DCM.

The trityl protecting groups were removed by incubating the resin twice for 15 min at 400 rpm with 4 mL of 10% (v/v) TFA, 2.5% (v/v) TIS and 87.5% (v/v) DCM. The resin was washed 5 times with 3 mL DCM. The trityl-deprotection step and the subsequent washing steps were performed on a TFA-compatible cleavage station.

The disulfide bridge was established by incubating the resin twice with 3 equiv. 2-pyridylthio cysteamine (1 mL, 0.15 M) in 30% (v/v) MeOH, 69.9% (v/v) DCM and 0.1% (v/v) AcOH for one hour at 400 rpm. 2-pyridylthio cysteamine was prepared as described below. The resin was washed twice with 3 mL 50% (v/v) DCM/ 50% (v/v) MeOH and twice with 3 mL DCM. For ensuring deprotonation of the cysteamine amino group, the resin was

incubated with 200 μL 4 M NMM in DMF for 1 min and washed with DMF. The two variable amino acids were introduced using the coupling conditions described above.

The bromoacetyl group was introduced by incubating the resin three times with 5 equiv. bromoacetic acid (0.6 mL, 0.5 M) and 5 equiv. *N,N'*-diisopropylcarbodiimide (0.6 mL, 0.5 M) in DMF for 30 min at 400 rpm. The resin was washed three times with 2 mL DMF followed by two times with 0.6 mL of DCM.

The protecting groups were removed and the peptides cleaved from the resin by incubating in 5 mL of 95% (v/v) TFA, 2.5% (v/v) mQ water and 2.5% (v/v) TIS for 90 min at 400 rpm. The cleavage was performed on a TFA-compatible cleavage station. Around 4 mL of the TFA-cleavage solution was evaporated under a flow of nitrogen. The peptides were precipitated in 50 mL cold diethyl ether for 1 hr at -20°C and centrifuged at 3,400 × g (around 4,000 rpm on a Thermo Scientific Multifuge 3L-R centrifuge with Sorval 75006445 rotor) for 10 min at 4°C. The supernatant was discarded.

For HPLC-purification, the solid was dissolved in 10% (v/v) ACN, 89.9% (v/v) mQ water and 0.1% (v/v) TFA and run over a reverse phase C18 column (Waters Sunfire™, 10 μm, 100 Å, 19 × 250 mm) using a linear gradient of solvent B (99.9% [v/v] ACN, 0.1% [v/v] TFA) over solvent A (99.9% [v/v] mQ water, 0.1% [v/v] TFA) from 10 to 34% in 34 min at a flow rate of 20 mL/min. The mass of the purified peptides was determined by electrospray ionization mass spectrometry using a Shimadzu-2020 single quadrupole LC-MS instrument. Fractions with the desired peptides were lyophilized. White powders were obtained and considered to be TFA salts.

Synthesis of 2-pyridylthio cysteamine

Cysteamine (1 equiv. in 30 mL MeOH, 0.39 M), 2,2'-dipyridyldisulfide (2 equiv., in 70 mL MeOH, 0.77 M) and AcOH (2 ml) were incubated overnight at RT under agitation. After removing the solvent by rotation evaporation, the 2-pyridylthio cysteamine product was precipitated by addition of 50 mL of cold diethyl ether, incubated for 30 min at -20°C and pelleted by centrifugation at 3,400 × g for 5 min at 4°C (around 4,000 rpm on a Thermo Scientific Multifuge 3L-R centrifuge with Sorval 75006445 rotor). The product was further purified in 7 cycles of dissolving the precipitate in 5 mL of MeOH and precipitation with 45 mL of cold diethyl ether and centrifugation. After the final centrifugation step, the white powder was dried by lyophilization. The resulting product was considered to be a chloride salt.

LC-MS analysis

Peptides and macrocycles were analyzed by a UHPLC and single quadrupole MS system (Shimadzu-2020). Samples were applied on a reversed-phase C18 column (Phenomenex Kinetex®, 2.6 μm, C18, 100 Å column, 50 × 2.1 mm) using a linear gradient of solvent B (99.95% [v/v] ACN and 0.05% [v/v] formic acid) over solvent A (99.95 % [v/v] mQ water and 0.05%[v/v] formic acid) from 0 to 100 % in 10 min at a flow rate of 1 ml/min using positive and negative modes for mass analysis.

Analytical HPLC

The purity of the peptides and macrocycles was analyzed by reversed-phase analytical HPLC (Agilent Technologies, 1260 Infinity). Fractions eluted from preparative HPLC (100 μL) were run over a reversed-phase C18 column (ZORBAX 300SB-C18, 5 μm, 4.6 mm × 20 mm) using a linear gradient of solvent B (99.9% [v/v] ACN, 0.1 % [v/v] TFA) over solvent A (94.9% [v/v] mQ water, 5% [v/v] ACN, 0.1% [v/v] TFA) from 0 to 100% in 15 min at a flow rate of 1 mL/min.

Macrocycle synthesis by acoustic liquid transfer

Acoustic liquid transfer was performed on an ECHO 550 from Labcyte/Beckman Coulter at the Biomolecular Screening Facility (BSF) of EPFL. All transfers were performed using DMSO transfer settings. ECHO-qualified 384-well low dead volume plates were used as source plates for peptides and amines. ECHO-qualified 384-well polypropylene plates (Labcyte 384 PP, Cat. # PP-0200) were used as source plates for linker reagents. Low volume 384-well plates (ThermoFischer NUNC™ 384 shallow well std height plates non-sterile, black, no lid; Cat. # 264705) were used as destination plates.

The building blocks were prepared in the following solvents and at the following concentrations, and transferred to 384-well source plates. *N*$^{\alpha}$-bromoacetyl peptide linear precursors was prepared at 10 mM in 80% (v/v) DMSO and 20% (v/v) mQ water, and 10 μL were transferred to a 384-well low dead volume plate. Amines were prepared at 160 mM in DMSO and 10 μL were transferred to a 384-well low dead volume plate. Primary amines purchased as HCl salts were supplemented with NaOH (200 mM final concentration; 400 mM for amine **54**). Cyclization reagents were prepared at 128 mM in DMSO and 50 μL were transferred to an ECHO-qualified 384-well plate.

For the N-alkylation reactions, 40 nL of peptide (1 equiv.) and 40 nL of amine (16 equiv.) were transferred in droplets of 2.5 nL. The plates were sealed with a plastic foil, centrifuged for 2 min at RT to ensure complete droplet merging (130 × g, corresponding to 1,000 rpm in a Sigma 2-6 centrifuge with a 11121 rotor) and incubated for 1 hr at 37°C.

For the removal of the thiol protecting group (the disulfide-linked tag peptide), 3.87 μL of buffer containing 60 mM NH_4HCO_3, pH 8.0, and 414 μM TCEP (4 equiv.) were transferred from a 384-well source plate with a liquid handling workstation (Caliper Sciclone ALH3000) using a 384 pipetting head and 25 μL disposable plastic tips. The plates were sealed with a plastic lid, centrifuged to ensure proper mixing (130 × g, 2 min, as above), and incubated 1 h at 37°C.

For peptide cyclization, 50 nL of the bis-electrophile linker reagents (16 equiv.) were transferred to the destination plates. The plates were sealed with a plastic lid, centrifuged as described above, and incubated 2 hrs at 37°C. The reactions had volumes of 4 μL and contained 100 μM peptide, 100 μM Tag, 1.6 mM primary amines, 400 μM TCEP and 1.6 mM linker. The final solvent was 60 mM NH_4HCO_3 pH 8.0 containing 3.05% (v/v) DMSO.

For HPLC and mass spectrometric analysis of test reactions performed with peptide BrAc-Trp$^{6\text{-Cl}}$-Ala-Cys*-S-S-Tag, 2-furanmethanamine, and linker reagents **1** to **7**, identical reactions were performed at the same scale as described above in 24 independent wells, the products pooled, and analyzed by analytical HPLC (85 μL of pooled sample) or LC-MS (10 μL of pooled sample) as described above. Yields and quantities of side products were determined from peak analysis by LC-MS at 220 nm considering area under the peaks of peptide derived products.

Cloning of MDM2 expression vector

The following DNA coding for the amino acids 17 to 125 of human MDM2 (hMDM2; in bold), containing also suitable DNA restriction sites (underline) for cloning into the bacterial expression vector pGEX-4T3 and expression as GST fusion, and sequences for primer annealing (italics), was ordered from Eurofins.

*5'-ATTCTATGCGGCCCAGC*GGATCC**AGCCAGATTCCGGCAAGCGAACAAGAAACCCTGGTTCGTC CGAAACCGCTGCTGCTGAAACTGCTGAAAAGCGTTGGTGCACAGAAAGATACCTATACCATGAAAGAGGT GCTGTTTTATCTGGGCCAGTATATTATGACCAAACGCCTGTATGATGAGAAACAGCAGCATATTGTGTATT GCAGCAATGATCTGCTGGGTGACCTGTTTGGTGTTCCGAGCTTTAGCGTTAAAGAACACCGTAAAATCTAT ACCATGATCTATCGTAATCTGGTGGTGGTTAATCAGCAAGAAAGCAGCGATAGCGGCACCAGCGTTAGCG AAAAC**TAAGAATTC*GGTTCTGGCGCTGAAA*-3'

The DNA was amplified by PCR using Phusion polymerase using the forward primer 5'-ATTCTATGCGGCCCAGC-3' and the reverse primer 3'-GGTTCTGGCGCTGAAA-5', and the DNA purified with a PCR purification kit (QIAquick® MinElute ® PCR Purification Kit, Qiagen).

The hMDM2 DNA (3.7 μg) and vector pGEX-4T3 (9.3 μg) were digested using *BamH*I (4 μL, 20,000 U/ml) and *EcoR*I (2 μL, 20,000 U/ml) in *EcoR*I buffer for 2 hrs at 37°C in a volume of 100 μL. The digested vector was additionally treated with FastAP phosphatase (13 μL, 1 U/μL) for 10 min at 37°C to prevent circularization. The cleaved PCR product and vector DNA were electrophoresed on an agarose gel using 1% (w/v) high quality agarose and TAE buffer containing 0.5 μg/mL of ethidium bromide and 1 mM guanosine. DNA products with the desired size were cut out using a scalpel under UV light (254 nm, 70% intensity) and purified by a gel extraction kit (QIAquick® Gel Extraction Kit, Qiagen). Concentrations were determined by measuring the absorption at 260 nm (NanoDrop 8000, Thermo Scientific).

Cleaved hMDM2 gene (443 ng) and pGEX-4T3 vector (446 ng) were ligated using T4 ligase (2 μL, 400,000 U/ml) in T4 ligase buffer in a volume of 21.2 μL for 4 hrs at RT. Completion of the ligation was analyzed by agarose electrophoresis and purified with a PCR purification kit.

The ligated vector (1 μL) was electroporated into 100 μL electrocompetent DH5$^{\alpha}$ *E.coli* cells using 1 mm electroporation cuvettes (EP-201, Cellprojects) and an electroporation device (MicroPulser, BIO-RAD). After electroporation, 1 mL of LB (Luria Broth) medium was immediately added to the cuvette, transferred to a sterile 14 mL tube, and incubated for 1 hr at 37°C under agitation (250 rpm). 100 mL of the culture were plated on LB agar plates containing ampicillin (100 μg/mL). After incubation overnight at 37°C, single colonies were picked with sterile plastic tips to inoculate 5 mL of LB containing 100 μg/mL ampicillin in 50 mL Falcon tubes. After overnight incubation at 37°C and agitation at 250 rpm, cells were pelleted by centrifugation at 10,000 × g (around 11,000 rpm on an Eppendorf Centrifuge 5418) for 5 min at 4°C. Plasmid DNA was isolated using a plasmid miniprep kit (NucleoSpin®

Plasmid Miniprep Kit, Qiagen). The DNA was sequenced (Microsynth AG) and a clone with the desired sequence was identified.

Sequence of hMDM2-GST

The DNA sequence was confirmed by Sanger sequencing using the primer Gex-5-for (5'-CCAGCAAGTATATAGCATGG-3') as is shown in Figure S5a. Expression in *E.coli* yielded the GST-hMDM2 fusion protein shown in Figure S5b.

Recombinant expression of human GST-hMDM2

The vector for expressing GST-hMDM2 was inserted into electrocompetent *E.coli* BL21 (DE3) cells by electroporation and plated on agar plates containing ampicillin (100 μg/mL). A 5 mL culture containing LB medium and ampicillin (100 μg/mL) was inoculated with cells of a single colony and incubated overnight at 37°C with agitation at 250 rpm. A culture flask containing 1 L LB medium and ampicillin (100 μg/mL) was inoculated with 5 ml cells of the pre-culture and incubated at 37°C under agitation at 250 rpm until OD_{600} reached 0.8 (around 4 h). The culture was cooled on ice for 10 min and protein expression induced by addition of IPTG to a final concentration of 1 mM. The culture was incubated for 5 hrs at 25°C under agitation at 250 rpm. The culture was centrifuged for 5 min 5,500 × g (around 5,000 rpm on a Thermo Scientific SORVALL RC BIOS centrifuge with a Fiberlite F8-6x1000y rotor) at 4°C. The supernatant was discarded, the pellet transferred to a 50 mL Falcon tube, flash-frozen in liquid nitrogen, and stored at -80°C.

The pellet was thawed on ice, suspended in cold lysis buffer containing 10 mM Na_2HPO_4, 1.8 mM $NaH_2PO_4*H_2O$, 137 mM NaCl, 2.7 mM KCl and 4 mM DTT, pH 7.4, supplemented with lysozyme (100 μg/mL), DNaseI (13 U/ml) and PMSF (0.2 mM). After 30 min incubation on ice, the cells were sonicated (SONICS Vibra cell; Method: Pulse 15 s on/ 45 s off, Amp 35%, 10 min) and centrifuged for 1 hr at 15,000 × g (around 10,000 rpm on a Beckman Coulter™ Allegra™ 25R using a TA-10-250 rotor) at 4°C. The supernatant was collected in a 50 mL Falcon tube and run over two connected 1 mL glutathione sepharose columns (GSTrap FF, GE Life Sciences) using a peristatic pump (BIORAD Econo Pump) at a flow rate of 1 mL/min. After washing with 10 column volumes of cold washing buffer (10 mM Na_2HPO_4, 1.8 mM $NaH_2PO_4*H_2O$, 137 mM NaCl, 2.7 mM KCl and 4 mM DTT, pH 7.4), the protein was eluted with 5 column volumes of elution buffer (50 mM Tris-HCl, pH 8.0, 4 mM DTT and 20 mM reduced glutathione). Protein in the fractions was denatured and reduced with loading buffer (5-fold loading buffer containing 2% [w/v] SDS, 25% [v/v] glycerol, 0.05% [w/v] bromophenol blue, 60 mM Tris-HCl pH 6.8 supplemented with pure β-Mercaptoethanol to a final concentration of 10 mM prior use), analyzed by SDS-PAGE using a 12% (w/v) RunBlue SDS protein gel and standard SDS running buffer (40 mM tricine, 60 mM TRIS, 0.1% (w/v) SDS, 2.5 mM sodium meta bisulfite, pH 8.4). The proteins in the gel were stained for 2 hrs with 0.1% (w/v) Coomassie R-250 in 50% (v/v) methanol, 10% (v/v) glacial acetic acid and 40% (v/v) mQ water, and de-stained overnight using de-staining solution containing 50% (v/v) mQ water, 40% (v/v) methanol and 10% (v/v) acetic acid. The fractions containing pure fusion protein were combined, the volume reduced by concentration to around 2 mL, and buffer exchanged with cold PBS by 5 cycles of adding 20 mL PBS and centrifugational concentration at 3,400 × g (around 4,000 rpm

with a Thermo Scientific Multifuge 3L-R centrifuge and a Sorval 75006445rotor) at 4°C with a Macrostep® Advance Centifugal Device having a 10,000 MWCO.

The protein concentration was determined by measuring absorbance at 280 nm ($MW_{GST\text{-}MDM2}$ = 38.8 kDa, $\varepsilon_{280\ nm}$ = 53'290 $M^{-1}cm^{-1}$). Protein purity was assessed by SDS-PAGE as described above. Around 80 aliquots of 50 μL with a concentration of 1.39 mg/mL were prepared, flash-frozen with liquid nitrogen, and stored at -80°C.

Synthesis of fluorescein-labeled MDM2-binding peptide

A peptide with the amino acids 15-29 of p53 (SQETFSDLWKLLPEN) labeled with fluorescein at the N-terminus spaced by a linker (GSGS) was synthesized as follows. The peptide GSGSSQETFSDLWKLLPEN-NH_2 was synthesized by standard Fmoc solid-phase chemistry at a scale of 25 μmol on Rink amide MBHA resin (loading: 0.3 mmol/g). In the last step of synthesis, 5(6)-carboxyfluorescein was coupled to the N-terminus as described before. The peptide was cleaved, precipitated with diethyl ether, and purified by RP-HPLC.

Measuring binding affinity of reporter peptide by fluorescence polarization

The binding of the reporter peptide was determined by incubating the peptide (50 nM final concentration) with 2-fold dilutions of GST-hMDM2 (form 25 to 0.01 μM final concentration) in assay buffer (10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4*H_2O, 137 mM NaCl, 2.7 mM KCl, pH 7.4, 0.01% [v/v] Tween-20) in a 384 well plate (ThermoFischer NUNC™ 384 shallow well std height plates non-sterile, black), incubation for 10 min at RT, and measuring fluorescence anisotropy (Infinite F2000 pro TECAN, E_{ex}= 485 nm, E_{em} = 535 nm, 25°C). The G-factor was calculated using 15 μL of fluorescein at 50 nM in assay buffer as a reference (minimum of polarization), and assay buffer as a blank. The K_d was calculated using Graphpad Prism 5 using the following formula (1):

$$y = a + (b - a)\frac{(K_d + x + P) - \sqrt{(K_d + x + P)^2 - 4}}{2P} \quad (1)$$

Where y is the anisotropy, a the probe signal in absence of fluorescent ligand (blank), b probe signal in the presence of saturating concentrations of ligand (reference), x and P the protein and probe concentrations respectively.

Establishing fluorescence polarization competition assay

For establishing the MDM2 fluorescence polarization competition assay, we synthesized the MDM2-binding peptide described above but without fluorescein (Ac-GSGSSQETFSDLWKLLPEN-NH_2) to use it as a control that was expected to displace the fluorescent probe (control peptide). Fluorescent reporter peptide (50 nM final concentration) bound to around 70% by GST-MDM2 (1 μM final concentration) were incubated with 2-fold dilutions of control peptide (20 to 0.02 μM final concentration) in assay buffer in a 384 well plate, incubated for 30 min, and fluorescence anisotropy measured as described above. The fluorescent reporter peptide and the control peptide were initially dissolved in DMSO, which added a small volume of DMSO. All wells were therefore complemented with DMSO to reach a final concentration of 1%.

The data were normalized considering negative control (no control peptide) and reporter probe alone (no protein) as 0 and 100% displacement of the reporter peptide, respectively. Sigmoidal curves were fitted to the data using Graphpad Prism 5 software and the following dose-response equation (2):

$$y = \frac{100}{1 + 10^{(\log IC_{50} - x)p}} \qquad (2)$$

Where y = displacement of reporter probe (%), x = peptide concentration and p = Hill slope. IC_{50} indicates the functional strength of the inhibitor (concentration resulting in 50 % displacement of reporter probe) and were derived from the fitted curve.

Screening macrocycle compound library

Macrocycles were synthesized in 384 well plates using ADE exactly as described above. To eight dedicated wells of each 384 well plates, 4 μl of positive control peptide (37.5 μM) was added manually. To eight other wells, only reaction buffer, TCEP and DMSO were dispensed as negative control. The other wells were filled combinatorially with the reagents to assemble the macrocycles, wherein one or two peptides were fit into one 384 well plate, along with all amines and linkers.

GST-hMDM2 and the fluorescent reporter probe were diluted using screening buffer (100 mM Na_2HPO_4, 18.5 mM $NaH_2PO_4.H_2O$, 137 mM NaCl, 2.7 mM KCl + 0.01% [v/v] Tween-20, pH 7.4) and mixed. The dilutions were chosen so that concentrations of 1.36 μM and 68 nM, respectively, were reached. After incubation for 10 minutes, 11 μl of the reagent mix was added to the each well of the assay plates using a bulk microplate dispenser (MultiFlo, BioTek) and a 1 μl cassette. Final volumes were 15 μl/well containing 1 μM GST-hMDM2, 50 nM fluorescent reporter peptide, and 26.7 μM macrocycle (assuming 100% yield). Final DMSO content was 0.8% (v/v). Final concentration of positive control was 10 μM. A plastic lid was manually added and the plates were incubated for 30 min at RT. The fluorescence anisotropy was recorded as described above.

Calculating percent of reporter peptide displacement

The raw anisotropy data was converted into % of reporter peptide displacement using the following equation wherein A is anisotropy:

$$\text{Displacement of reporter peptide } (\%) = 100 \times \frac{\text{A (negative control)} - \text{A (macrocycle reaction)}}{\text{A (negative control)} - \text{A (positive control)}} \qquad (3)$$

The A (negative control) is the anisotropy in absence of a competitor. The A (positive control) is the anisotropy in absence of MDM2. On each 384-well plate, eight wells were dedicated for each one of the two controls and mean values were calculated and used in the equation above. For presenting the data in the heat maps shown in Figure 4b and Figure S7, the values (% of reporter peptide displacement) were converted into colors applying the color gradient shown in Figure 4b and using Excel.

Hit confirmation

Macrocycle hits showing strong displacement of reporter peptide were synthesized at a 10 nmol scale, which was 25-fold higher than in the screen. All reagents were transferred by pipetting to an Eppendorf tube which was centrifuged after each transfer. The following synthesis describes the procedures for the 10 nmol scale. The N_{α}-bromoacetyl peptides (1 μl, 10 mM, 80% [v/v] DMSO and 20% [v/v] mQ: H_2O) were mixed with primary amines (1 μl, 160 mM in DMSO) and incubated at 37°C for 1 h. A volume of 96.7 μl TCEP (60 mM NH_4HCO_3, 414 μM TCEP, pH 8.0) was added and incubated at 37°C for 1 h for deprotecting the thiol group. The cyclization was performed by adding bis-electrophile reagent (1.25 μl, 128 mM in DMSO) and incubating for 2 h at 37°C. Negative controls were performed using the solvent and buffer only following the same protocol. The binding of the reaction products to hMDM2 was measured using the fluorescence polarization competition assay as described above.

Identification of active compounds in reactions

Macrocycle were synthesized at a 50 nmol scale essentially as described for the 10 nmol scale above, which was 125-fold higher than in the screen. The products obtained were separated on a reverse phase C18 column (Nova-Pak® C18, 6 μm, 7.8 x 300mm) using UHPLC (Thermo Scientific, UltiMate 3000, *Flow cell*: SST, 13 μL, 10 mm, 120 bar) and a linear gradient of solvent B (99.9% [v/v] ACN and 0.1% [v/v] TFA) over solvent A (99.9% [v/v] mQ:H_2O and 0.1% [v/v] TFA) from 0 to 80% in 30 min at a flow rate of 4 ml/min. Fractions of peaks were lyophilized and the white powders dissolved in 12.5 μL DMSO followed by addition of 238 μL of mQ: H_2O. The activity of the fractions was tested using the fluorescence polarization competition assay as described above.

Preparative macrocycle synthesis

Macrocycles were produced at a 25 μmol scale without the tag on cysteamine 4-methoxytrityl resin (Novabiochem®, loading: 0.92 mmole/g) and the N-alkylation step was performed on solid phase. Fmoc solid phase synthesis and bromoacetylation were performed as described above. Primary amines were incubated with the resin (10 equiv. in DMF, 0.5 mL, 0.5 M) for 1 h under agitation. The resin was washed three times with 3 ml DMF and twice with 3 ml of DCM. Cleavage and ether precipitation were performed as described above. The resulting white powder was solubilized in a small volume of DMSO, complemented with buffer (60 mM NH_4HCO_3, pH 8.0), and cyclized with 4 equiv. of bis-electrophile reagent. After 2 h incubation at 37°C, the mixture was lyophilized. The resulting solid powder was solubilized in 10% (v/v) DMSO, 20% (v/v) ACN, 69.9% (v/v) mQ: H_2O and 0.1% (v/v) TFA, and purified by RP on a C18 column (Nova-Pak® C18, 6 μm, 7.8 x 300mm) by UHPLC (Thermo Scientific, UltiMate 3000, *Flow cell:* PEEK, 0.7 μL, 0.4 mm, 100 bar) using a linear gradient of solvent B (99.9% [v/v] ACN and 0.1% [v/v] TFA) over solvent A (99.9% [v/v] mQ: H_2O and 0.1% [v/v] TFA) from 20 to 70% in 30 min at a flow rate of 4 ml/min. After lyophilization the macrocycles were dissolved in DMSO and adjusted to a concentration of 10 mM. The mass of the purified peptides was determined by electrospray ionization MS (ESI-MS) as described above.

Measuring macrocycle binding by SPR

The binding affinity of macrocycles for immobilized MDM2 was measured by surface plasmon resonance (SPR). The experiments were performed using a BiacoreTM 8K instrument (GE Healthcare). GST-hMDM2 fusion protein (10 μg/mL) was dissolved in 10 mM MES buffer (pH 6.0) and immobilized on a CM5 series S chip by a standard amine coupling method in running buffer (10 mM Na_2HPO_4, 1.85 mM $NaH_2PO_4 \cdot H_2O$, 137 mM NaCl, 2.7 mM KCl and 0.005% (v/v) Tween-20). The immobilization level was around 3000 resonance units (RUs). The reference cell was treated the same way without GST-hMDM2. For the measurement of binding kinetics and dissociation constants, five serial dilutions (4-fold) of macrocycles were prepared in running buffer containing 0.4% (v/v) DMSO and analyzed in single cycle kinetics mode with the contact and dissociation times of 60 s and 120 s, respectively.

SUPPLEMENTARY FIGURES

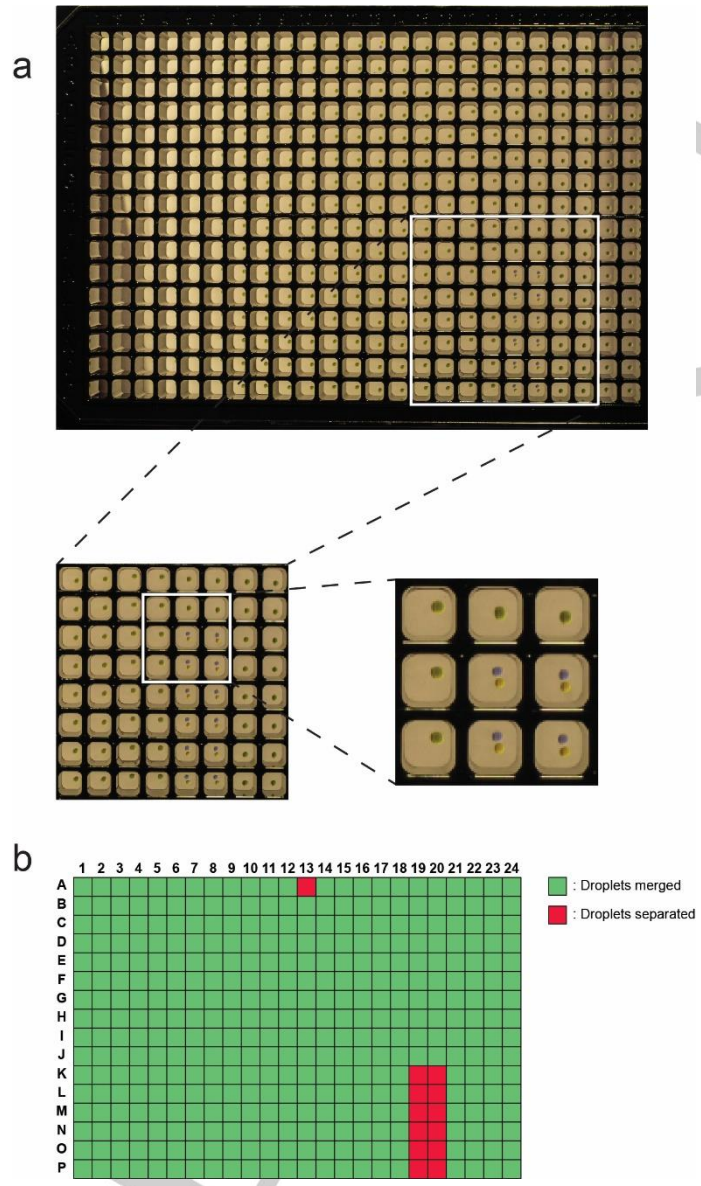


Figure S1. Merging of droplets transferred by acoustic dispensing in a 384-well microtiter plate. (a) Photo of a microtiter plate to which 20 nL Coomassie and fluorescein droplets were transferred. Below is shown an enlarged region in which the two droplets did not merge in four of the nine wells. (b) Indication of wells in which droplets were merged (green) or not merged (red).

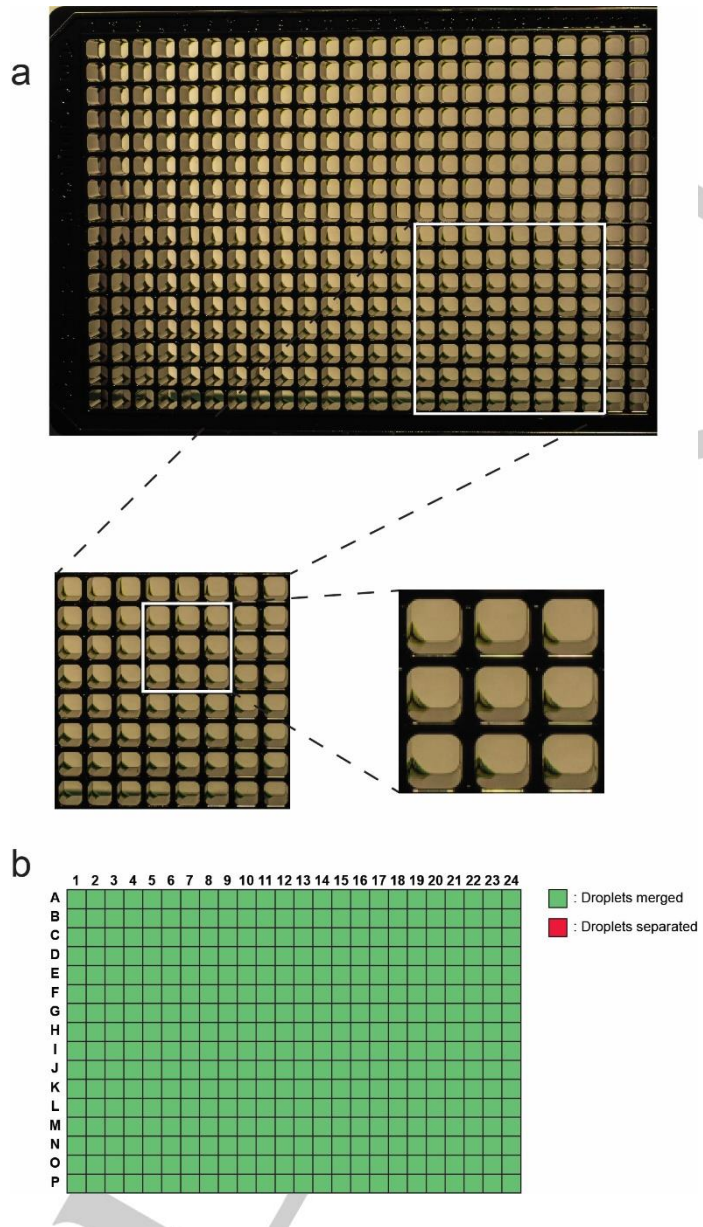


Figure S2. Enforcing droplet meeting in a microtiter plate by centrifugation. (a) Photo of the microtiter plate shown in Figure S1 after centrifugation and enlarged regions below. The merged liquids are adhered to the lower left corner of the wells. (b) In all wells, the droplets were merged (green).

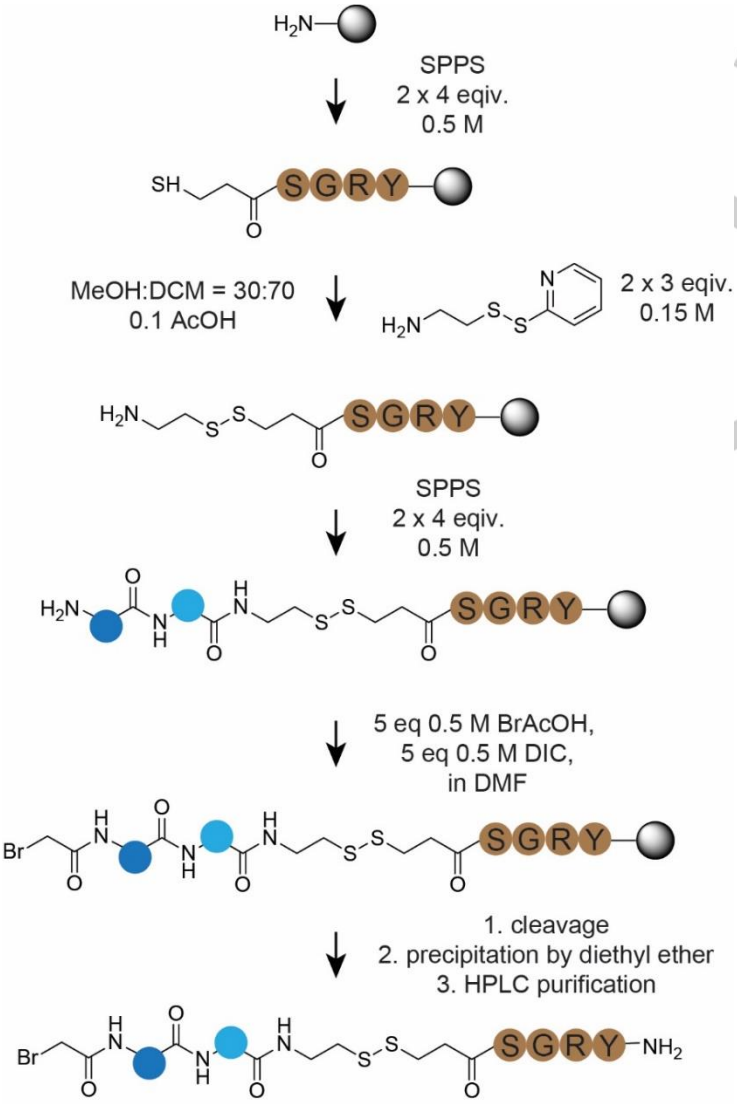


Figure S3. Synthesis of thiol-protected and bromoacetamide-functionalized peptides. Peptides were synthesized on solid phase using rink amide resin (50 μmol scale). They contain a C-terminal mercaptopropionic acid-Ser-Gly-Arg-Tyr tag that serves two functions, one facilitating purification of the peptides by precipitation with diethylether, and one protecting the peptides' thiol group by a disulfide bridge.

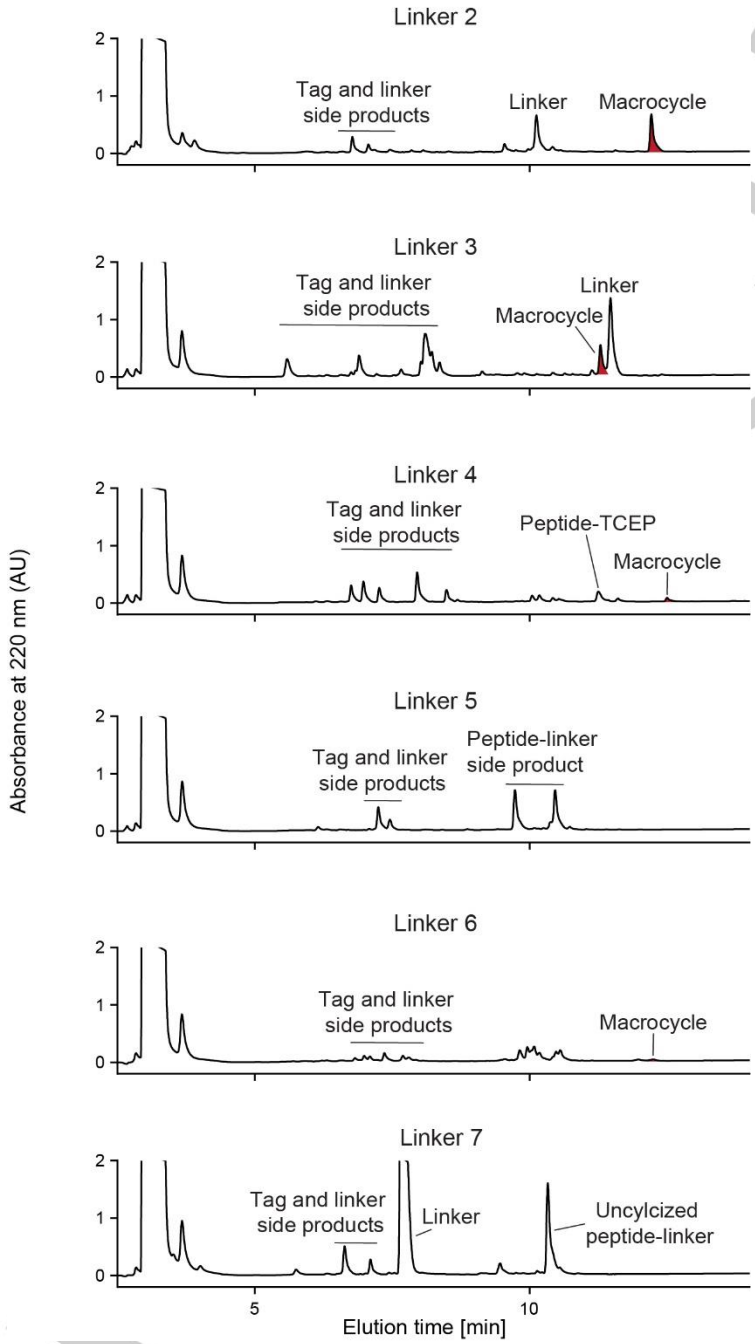


Figure S4. Thiol-to-amine macrocyclization by cyclization reagents 2-7. A BrAc-activated model peptide (Figure 2a) was reacted with the example primary amine 2-furanmethanamine, and cyclized by the bis-electrophile reagents **2**-**7** (indicated as "linker"). Reagents were transferred by ADE as shown in Figure 2a.

DNA sequence:

5'-ATCCTCCAAAATCGGATCTGGTTCCGCGTGGATCC**AGCCAGATTCCGGCAAGCGAACAAGAAA**
CCCTGGTTCGTCCGAAACCGCTGCTGCTGAAACTGCTGAAAAGCGTTGGTGCACAGAAAGATACCTATAC
CATGAAAGAGGTGCTGTTTTATCTGGGCCAGTATATTATGACCAAACGCCTGTATGATGAGAAACAGCAG
CATATTGTGTATTGCAGCAATGATCTGCTGGGTGACCTGTTTGGTGTTCCGAGCTTTAGCGTTAAAGAACA
CCGTAAAATCTATACCATGATCTATCGTAATCTGGTGGTGGTTAATCAGCAAGAAAGCAGCGATAGCGGC
ACCAGCGTTAGCGAAAACTAAGAATTCCCGGGTCGACTCGAGCGGCCGCATCGTGACTGACTGACGATCT
GCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGT
CTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGC
GCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATAATTCTTGAAGACGAAAGGGCCTCGTGATACG
CCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTG
CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATA
AATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTG
CGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGG
GTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAA
CGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCSGGGCA
AGAGCAACTCGGTCGCCGCATACACAAGT-3'

Protein sequence:

MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIAD
KHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPD
*FMLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSD*LVPRGS**S**
QIPASEQETLVRPKPLLLKLLKSVGAQKDTYTMKEVLFYLGQYIMTKRLYDEKQQHIVYCSNDLLGDLFGVPSF
SVKEHRKIYTMIYRNLVVVNQQESSDSGTSVSEN

Figure S5. DNA and protein sequence of GST-hMDM2. The region of the expression vector that was confirmed by Sanger sequencing is shown. In bold is the gene coding for hMDM2 (17-125 aa). The cleavage sites of the restriction enzymes BamHI and EcoRI are underlined. The stop codon is highlighted in red. Amino acid sequence of the GST-hMDM2 fusion protein. The amino acids of GST are shown in italics and those of hMDM2 (17-125 aa) in bold.

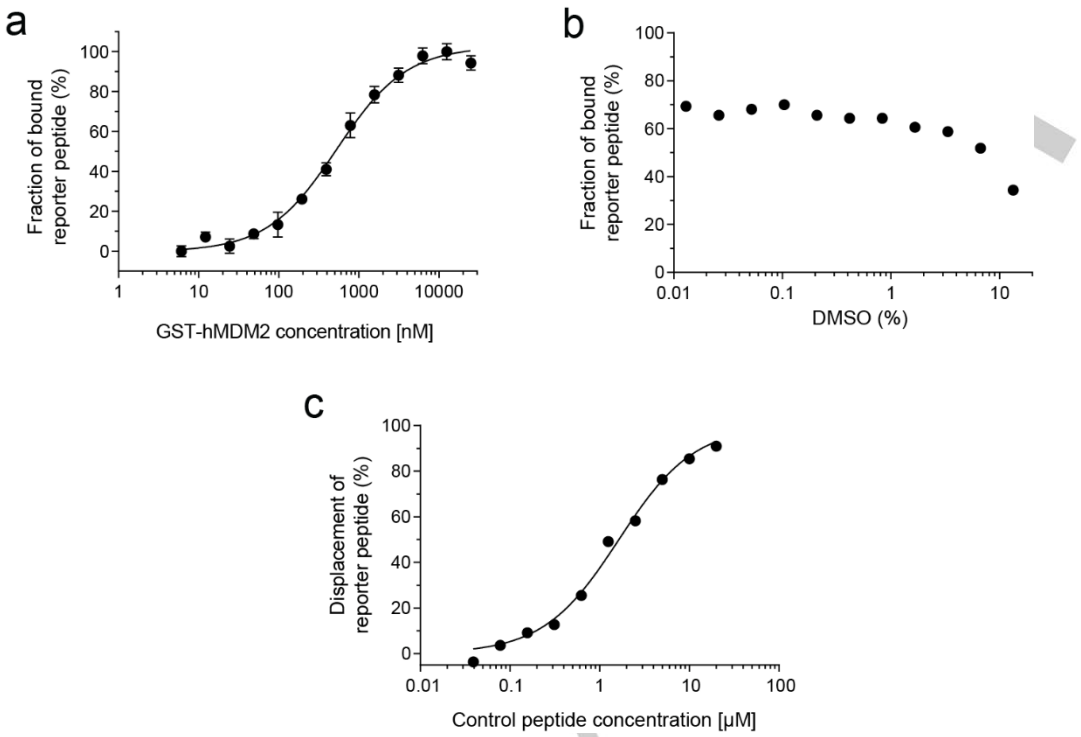


Figure S6. Fluorescence polarization assay development. (a) Binding of the fluorescent reporter peptide 5(6)-FAM-GSGSSQETFSDLWKLLPEN-NH_2 to GST-hMDM2 measured by fluorescence anisotropy. Means and standard deviations of three measurements are shown. (b) Effect of different DMSO concentrations on the binding of GST-hMDM2 (1 μM) to the fluorescent reporter peptide (50 nM) in the fluorescence polarization assay. Average values of two measurements are shown. (c) Fluorescence polarization competition assay. The displacement of the fluorescent reporter peptide (50 nM) from GST-hMDM2 (1 μM) at increasing concentration of the non-fluorescent control peptide was quantified by measuring fluorescence anisotropy.

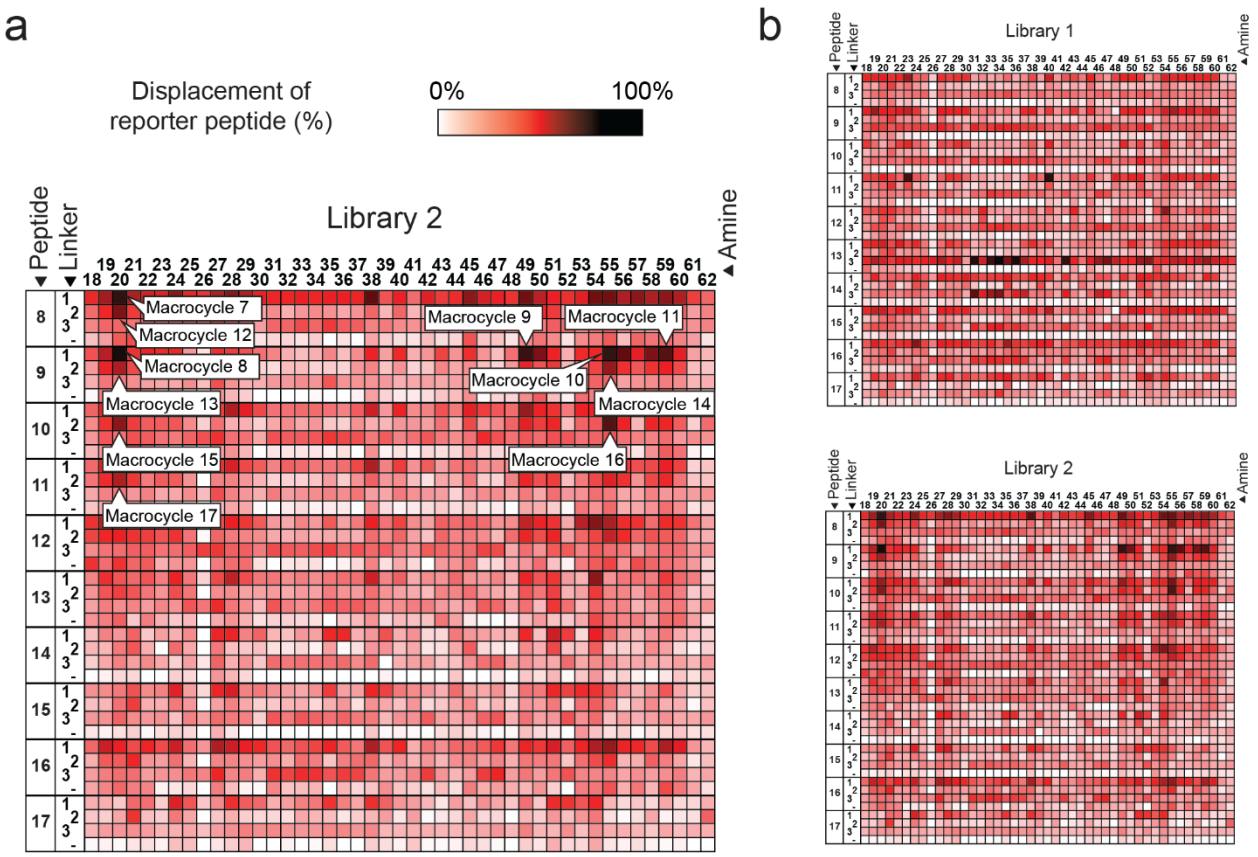
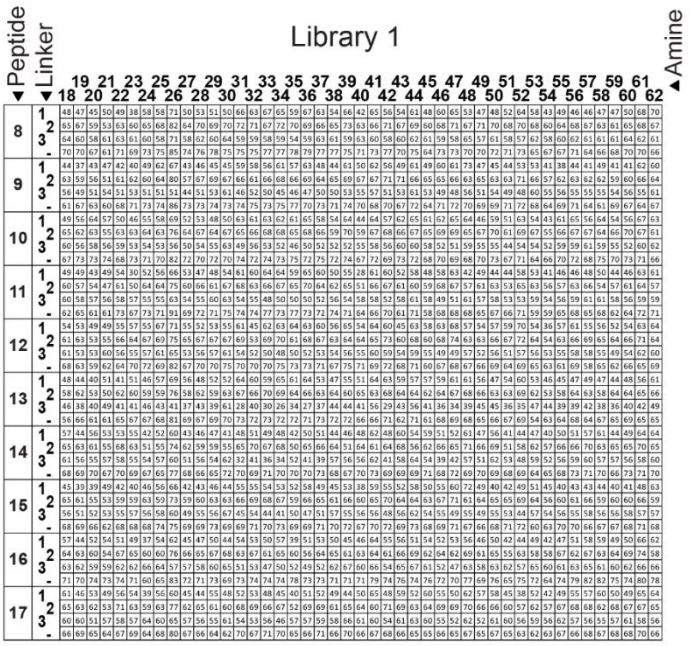


Figure S7. Screen for MDM2-p53 PPI inhibitors. (a) Binding of the 1,350 macrocycles of Library 2 to MDM2. The binding was assessed by displacement of a fluorescein-labeled MDM2-bound reporter peptide using fluorescence polarization. Darker colors indicate higher binding affinity. The building blocks used for library synthesis (shown in Figures 2c and 3) are indicated by number. Control reactions (linear peptides with no linker) are indicated by "-". Macrocycles that were re-synthesized, purified, and characterized are indicated on the heat map. (b) Heat map from screens with Library 1 and 2 without annotations. (c) Raw data of screen. The values indicate the measured anisotropy. The mean anisotropy of the negative controls (no competitor) and the positive controls (reporter peptide only) were 77 and 13, respectively. (d) Repetition of the screen for the 17 hit compounds and 16 randomly chosen reactions. The randomly chosen reactions are from Library 2 and are indicated with a black arrow in the top left panel. The bar graphs show the % of displaced reporter peptide for the screen and the three replicates.

C



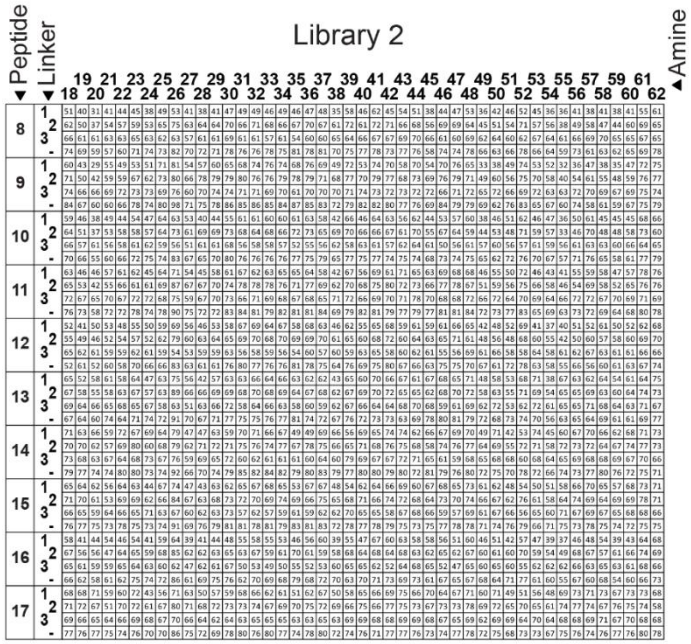


Figure S7. Continued

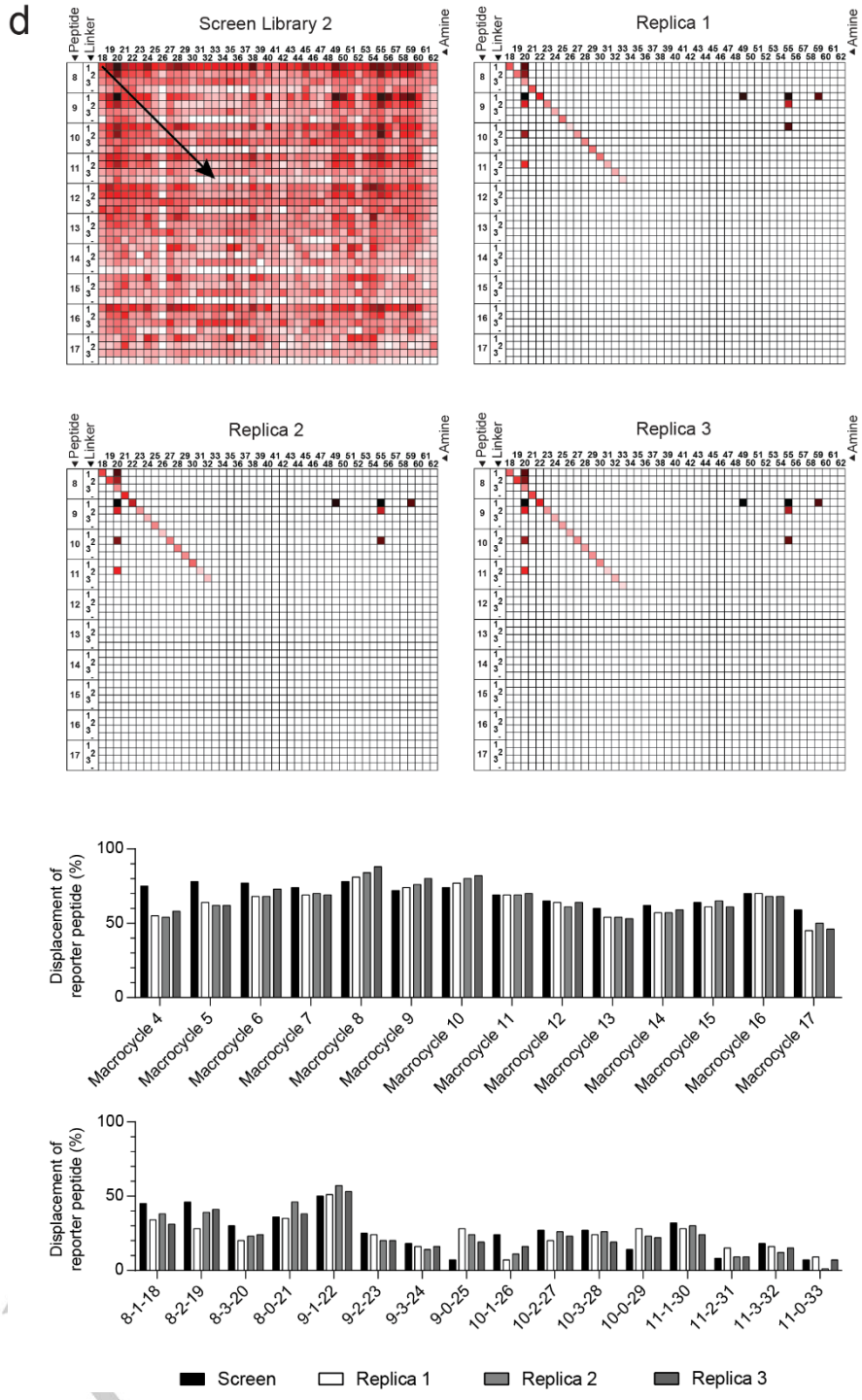


Figure S7. Continued

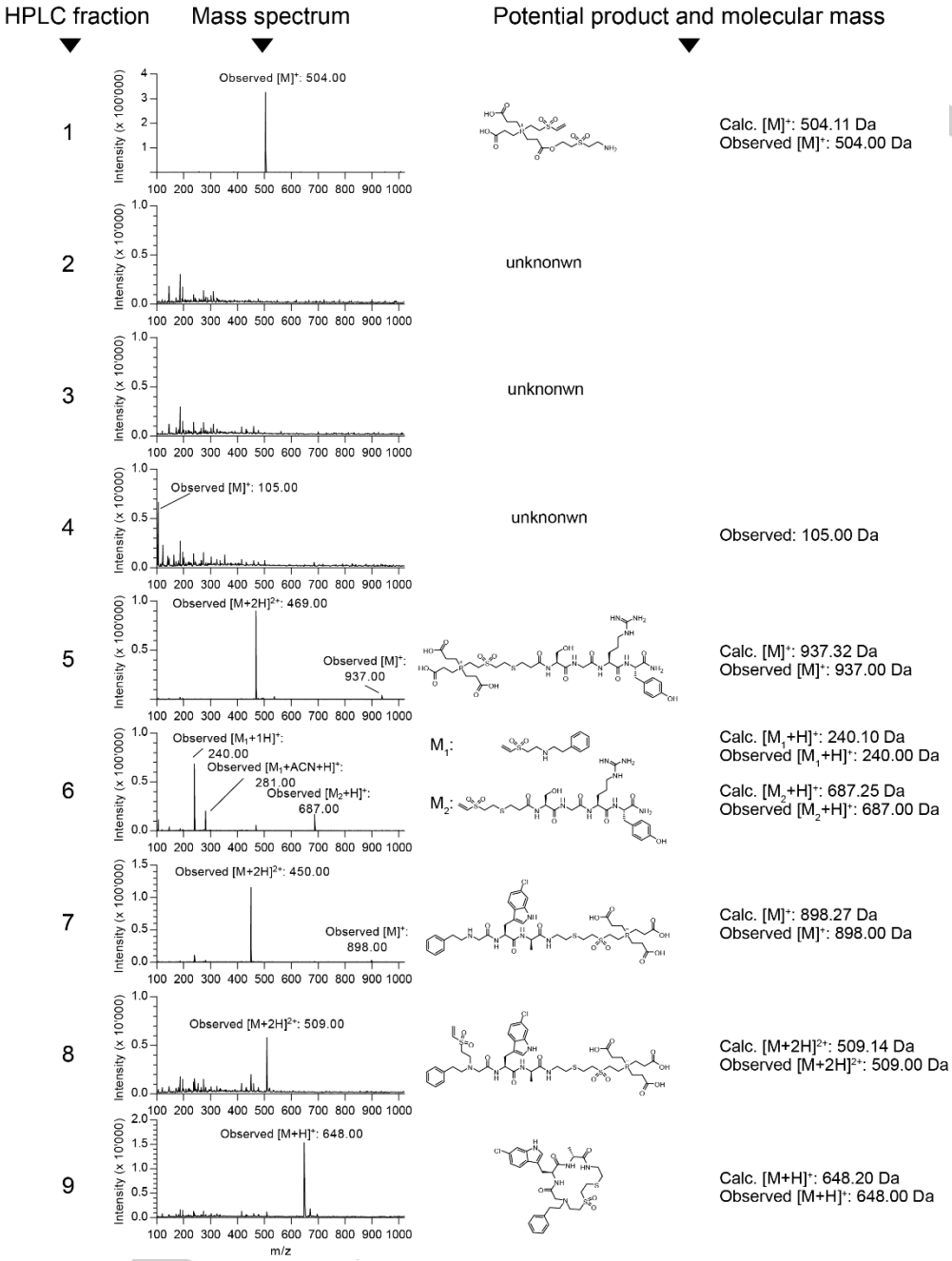


Figure S8. Side-products identified in the reaction mix of macrocycle 1. Macrocycle 1 was synthesized at a 50 nmol scale using the same "mixing and reaction" procedure that was used to prepare the library, the reaction run over a C18 column by RP-HPLC, and molecules that eluted as peaks shown in Figure 5c analyzed by ESI-MS. Peak 9 contains the macrocycle 1.

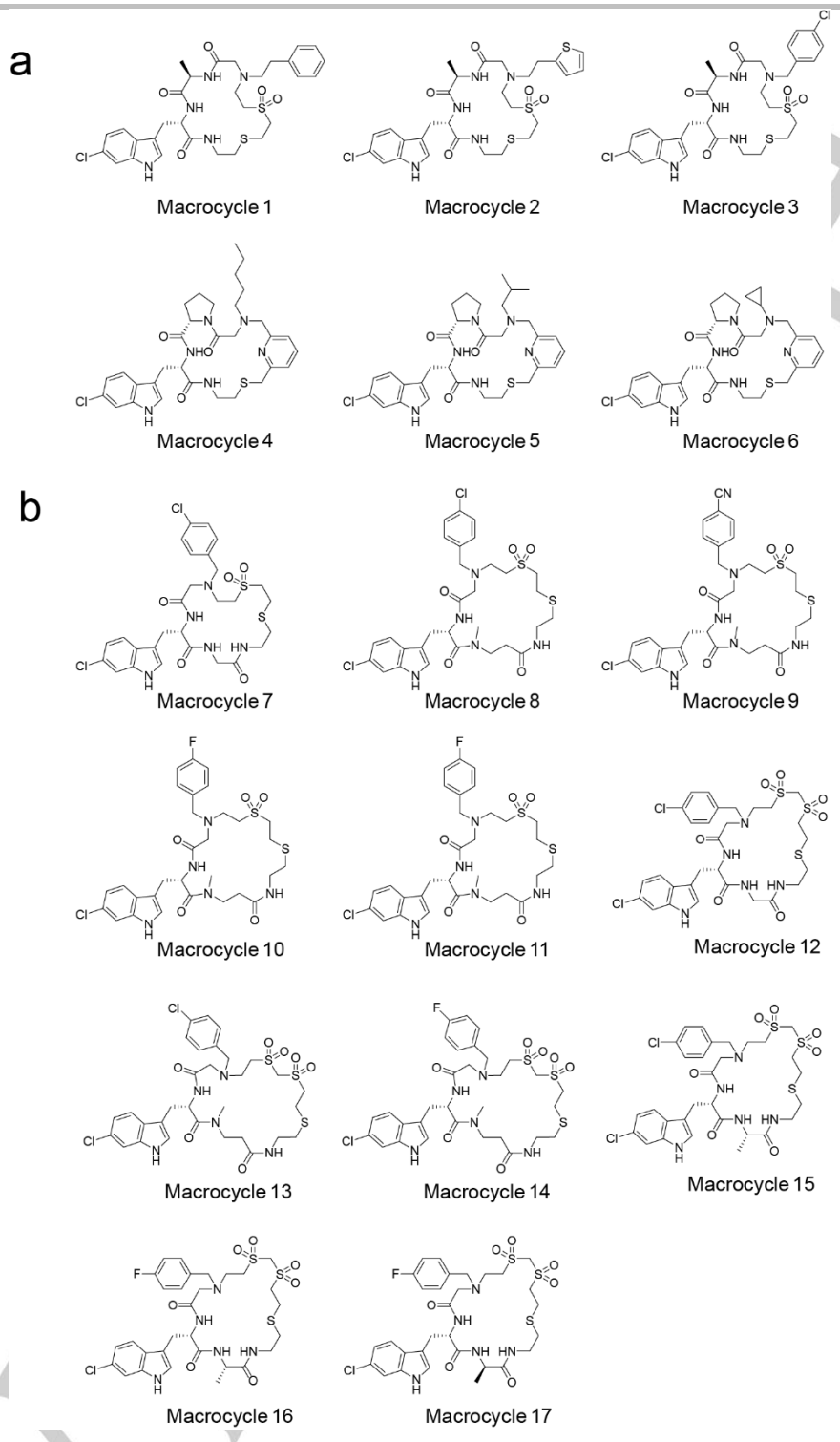


Figure S9. Chemical structures of macrocycles. Hits from Library 1 (a) and Library 2 (b).

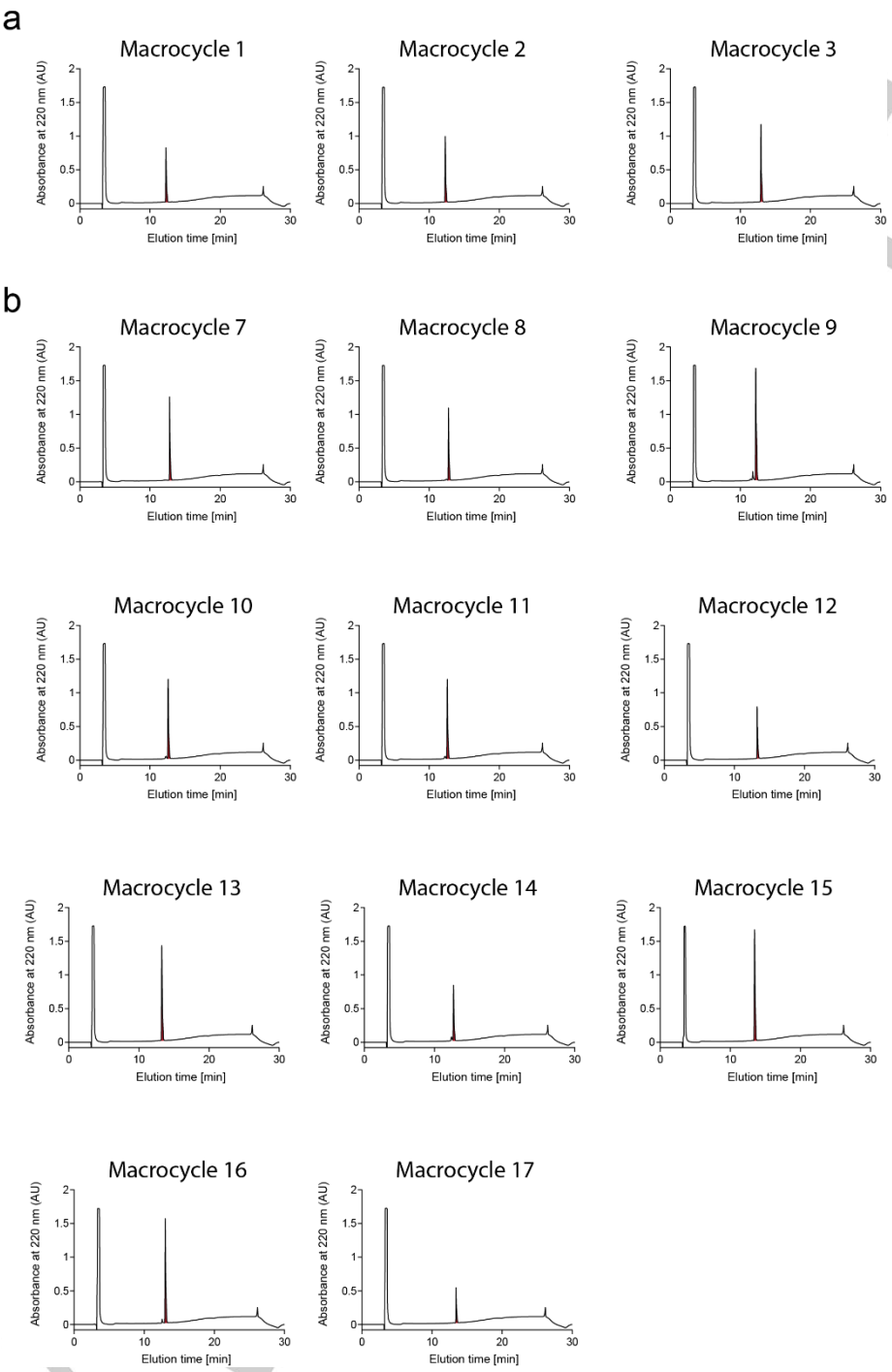
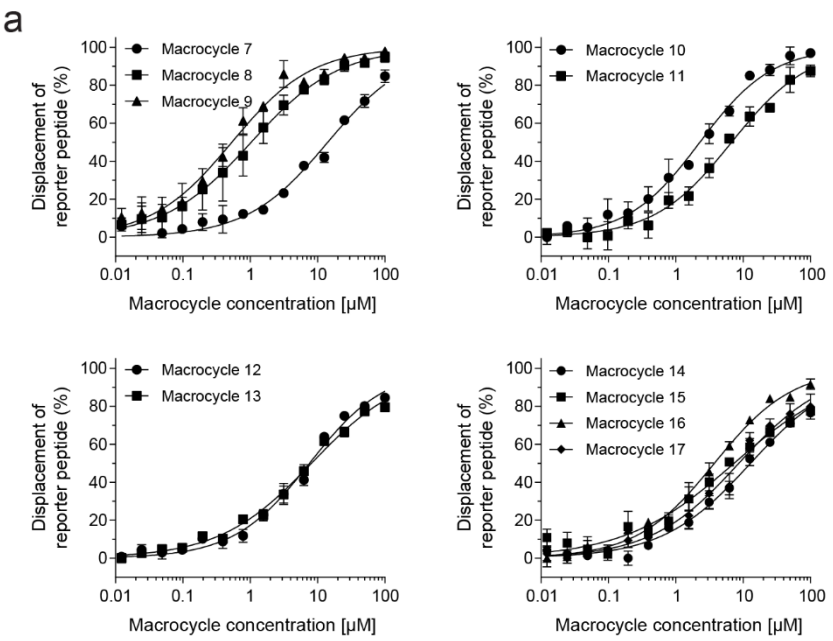
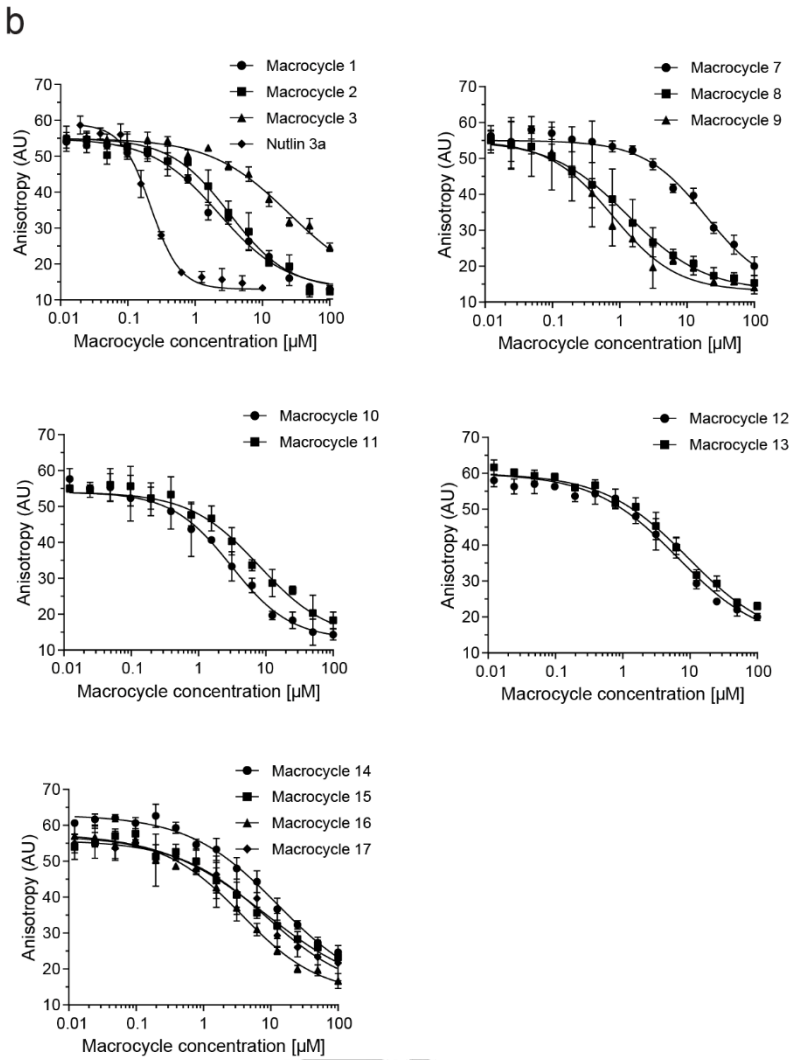


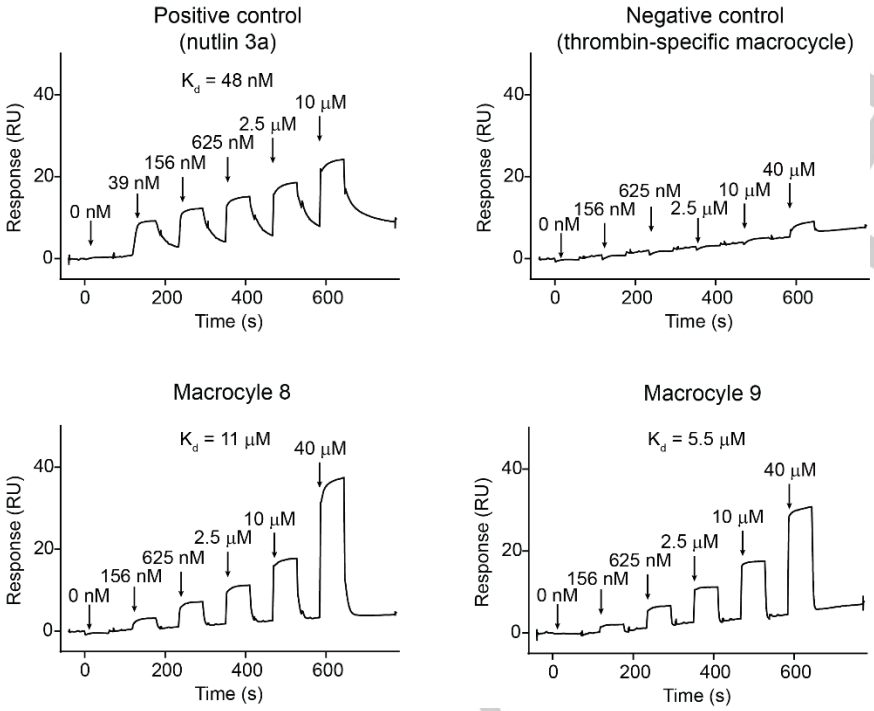
Figure S10. HPLC analysis of macrocycles. Hits from Library 1 (a) and Library 2 (b).



Supplementary Figure 11. Binding of purified macrocycles to MDM2. The binding to GST-hMDM2 was measured with the fluorescence competition assay. Mean values and standard deviations of three independent measurements are shown. (a) Displacement of reporter peptide in % calculated based on the change in fluorescence polarization, as described in the materials and methods. (b) Raw data of fluorescence polarization competition assay. The extent of polarization is indicated as anisotropy.



Supplementary Figure 11. Continued



Supplementary Figure 12. Measurement of macrocycle binding affinity by SPR. MDM2 was immobilized and macrocycles were run over the chip at the indicated concentrations. Single cycle SPR sonograms of the best two macrocycles and two controls are shown. The small molecule MDM2 binder nutlin 3a was used as a positive control. A macrocycle with similar size and format, binding to a different target (thrombin), was used as a negative control.