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ARTICLE

Screening for covalent inhibitors using DNA-display of small molecule libraries functionalized with cysteine reactive moieties†

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DNA-encode chemical libraries are increasingly used to identify leads for drug discovery or chemical biology. Despite the resurging interest in covalent inhibitors, libraries are typically designed with synthon filtered out for reactive functionalities that can engage a target through covalent interactions. Herein, we report the synthesis of two libraries containing Michael acceptors to identify cysteine reactive ligands. We developed a simple procedure to discriminate between covalent and high affinity non-covalent inhibitors using DNA display of the library in a microarray format. The methodology was validated with known covalent and high affinity non-covalent kinase inhibitors. Screening of the library revealed novel covalent inhibitors for MEK2 and ERBB2.

Introduction

Despite the notable success of covalent inhibitors in our pharmacopeia (β-lactam, aspirin, omeprazole, clopidogrel for instance), there is a historic reluctance to pursue the development of compounds containing mildly reactive functionalities that can engage a target through covalent interactions.[1] This is in contrast to natural products where evolutionary pressure for bioactivity has frequently yielded covalent inhibitors.[2] In addition to natural products targeting particularly activated residues within nucleophilic enzymes (proteases and hydrolases), there is a plethora of natural products that engage discrete nucleophilic residues in the vicinity of a ligand-binding site. For instance, helenalin, which is part of an extract of Arnica Montana, reacts with p65 thus inhibiting the NF-κB pathway[3] and is used topically to treat inflammation. Another inspiring example is that of the resorcylic acids that react with a discrete cysteine in the nucleotide binding site of a subset of kinases.[4] The challenge of achieving sustained efficacy with some targets has recently led to a resurging interest in covalent inhibitors,[5] and two covalent kinase inhibitors received FDA approval in 2013 (Afatinib, Iburtinib).[6] This rising interest in covalent inhibitors warrants methodologies to identify covalent inhibitors in a primary screen. In that respect, enzymatic assays are ill suited since they do not discriminate between a reversible and covalent inhibitor. DNA encoding technologies for small molecule libraries are increasingly used in the discovery of novel drug leads or probes for chemical biology.[7-10] The majority of the libraries aim to combine synthons that yield a final library with desirable drug-like properties.[11] These designs generally filter out potentially reactive functionalities.

Results and discussion

Several technologies have been developed to synthesize DNA-encoded libraries: DNA-templated synthesis, split and mix synthesis using DNA-tag ligation (enzymatic or chemical), or hybridization to route the DNA tags.[12-32] In addition, peptide nucleic acid (PNA) encoding has been pursued on the basis that its encoding chemistry utilizes SPPS that it is compatible with co-synthesis of peptides and small molecule libraries.[33-45] Several studies have established that hybridization of PNA-tagged molecules onto a DNA microarray can be used to rapidly identify the best binders within a library (Fig. 1).[36, 44-50] We reasoned that this method could allow us to discriminate between covalent and non-covalent ligands using a denaturing wash that would denature proteins and compromise non-covalent ligand-protein interactions. However, aside from peptide-based libraries targeting proteases,[33, 37-38] no DNA or PNA-encoded library specifically designed to engage diverse protein targets in covalent interactions had been reported. Herein we report two PNA-encoded libraries specifically designed to contain functionalities that can engage nucleophilic residues covalently.

The first library was designed to have two points of diversity with the second set of synthon including diverse Michael acceptors (library I, Fig. 2). This reactive moiety was selected based on the fact that it is present in many natural products and has been successfully used for designed covalent inhibitors targeting cysteine residues. The synthesis proceeded as shown in Scheme 1, using resin **1** loaded with the orthogonally protected (Fmoc/Mtt) bifunctional linker and a 12 Å PEG linker to distance the small molecule from its PNA

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tag. The resin was split in 100 pools, and loaded with 100 diverse Fmoc-protected amino acids (see Fig. 1 for representative examples). The Mtt group was removed (HFIP) and the structure of the diverse amino acids was encoded with a 7mer PNA. The quality of the synthesis was verified by a cleavage of an analytical sample of resin for MALDI analysis. The pools were combined and re-split into 100 pools. In order to avoid exposing the library containing the Michael acceptor to the encoding chemistry, the second codon was introduced first (7mer PNA terminated with a BocLys(Mtt)OH) and the Fmoc group on the amino acid was removed to introduce the synthon bearing the Michael acceptor; 34 synthons were obtained from commercial vendors while the rest were generated by coupling 2-methylenepentenoic acid followed by a CuAAC[51-52] with 2-azidoacetic acid and derivatization with 66 diverse amines. The 100 pools were subjected to analytical cleavage and MALDI analysis confirming that each pool had a molecular weight distribution that was consistent with a full synthetic conversion. The pools were mixed and the library was then cleaved from the resin to obtain the 10 000-membered-library in solution.

A second library was designed to target more specifically kinases based on the use of a core scaffold that binds the hinge domain of kinases (quinazoline or resorcylic acid moiety of known kinase inhibitors[53-56]): Library II, Fig. 3. Co-crystal structures of resorcylic acid lactones with kinases[57] have shown that the 2-hydroxybenzoic moiety occupies a comparable space and is involved in a similar network of interactions as the quinazoline motif in kinase inhibitors. The library was envisioned to have the same starting point as library I, Novapeg Rink resin bearing a Dap linker and a PEG spacer between the encoding region and the diversity region. The diverse scaffolds were planned to be connected to the first amino acid synthon using three different chemistries: nucleophilic aromatic substitution (S_NAr) for the quinazoline, classic amide coupling for the resorcylic acid analogs or a CuAAC[51-52] to obtain a triazole in lieu of the amide on the resorcylic acid scaffold. The last point of diversity was planned to arise from latent functionalities on the aryl moiety: aryl halide or suitably protected amines to be further derivatized by Suzuki-reaction or acylation with Michael acceptors respectively. Based on the fact that S_NAr reaction and Suzuki reaction had never been used in PNA-encoded library synthesis, all the synthons used were validated on the resin with a PNA tag to verify the efficacy of the chemistry (full synthetic details can be found in the Supporting Information). Starting with **1** (scheme 2), the resin was split into 100 pools and each one was reacted with a unique amino acid, the Fmoc group was then removed and the resulting amine converted to an azide using imidazole-1-sulfonyl azide hydrochloride (**7**) to obtain **8**. Each pool was encoded with a 7mer PNA (**9**) and the resins were combined and re-split into 100 new pools. Each of these pools was taken through a unique set of transformations to afford 100 combinations that were encoded with a 7mer PNA (see scheme 2 for structures of individual fragments). Thus, 52 out of the 100 pools were treated with tris(2-carboxyethyl)phosphine (TCEP) to reduce the azide. A subset of these 52 pools (25) was engaged in a S_NAr with diverse quinazolines (2 synthons) that were further each diversified by palladium-catalyzed Suzuki coupling with 10 and 15 diverse boronic acids respectively thus yielding 25 unique combinations. The remaining 27 pools were acylated with diverse resorcylic acids (5 synthons). The synthons bearing an Alloc-protected aniline were deprotected and further diversified with Michael acceptors. Alternatively to the azide reduction, 48 pools of resin **10** were engaged CuAAC couplings with diverse alkynes (5 synthons) and the products were further diversified through the aforementioned palladium cross couplings or acylation chemistry. In addition, one pool contained a benzylic chloride that was diversified by nucleophilic displacement with a thiophenol. Each of the pool was subjected to an analytical cleavage and MALDI analysis to confirm the execution of the chemistry. Pools that failed the quality control were re-prepared. The pools were mixed and the library cleaved from the resin to obtain a second library of 10 000 compounds with 27% of the library bearing a reactive functionality targeting cysteines. In addition, we prepared four known kinase inhibitors conjugated to PNA (a covalent EGFR/ERBB2 inhibitor: canertinib;[58] two high affinity but nocovalent pankinase inhibitor: dasatinib,[59] a staurosporin-based bisindole maleimide (BIM);[60-61] and high affinity allosteric inhibitor of MEK: PD-0325901[62], see Fig. 4 for structures) as controls to establish the screening protocol. For the controls, the PNA tag was functionalized with a Cy3 fluorophore to visualize its hybridization and ensure that the compound is present on the microarray upon spiking within a library. The Cy3 fluorophore used is spectrally resolved from the fluorophore (Dylight 649) used in protein detection (Cy5 channel).

In order to establish the conditions to discriminate between high affinity ligands and covalent ligands, we first investigated a screen against a therapeutically relevant kinase (ERBB2) and library I (Fig. 2) based on the availability of a control that binds covalently to this kinase (canertinib) and a high affinity pankinase ligand (BMI). The array was self-assembled by hybridization of the PNA-encoded library spiked with both controls onto a DNA microarray (Agilent custom array, design 048196) under standard conditions.[36, 44] The array was then treated with the His-tagged ERBB2 in buffer (50 mM Hepes pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.05% Tween 20) for 60 min, washed with the same buffer and treated with a Dylight 649 anti-HisTag antibody. A scan in the Cy3 channel confirmed that the controls were at the preprogrammed position based on the complementarity of their PNA tags with the DNA sequence on the array. A scan in the Cy5 channel indicated that there were several binders, including the two positive controls: the covalent inhibitor (canertinib: position: 1-2, Fig. 4A) and high affinity but non-covalent ligand (BMI: position 79-75, Fig. 4A). In parallel, the same screen was performed followed by a wash using more stringent conditions with a denaturing buffer (PBS, 1% SDS for 15 min with gentle rotation), Fig. 4B. It is important to note that despite the stringent conditions, the PNA-DNA interaction remains unaffected and this point was verified by comparing the Cy3

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intensities corresponding the controls. Comparison of the intensity of the signal of the two controls in the Cy-5 channel revealed a significant loss of intensity for the non-covalent control (BIM) using the stringent wash while the covalent control retained its intensity (six fold difference in the ratio of fluorescence intensity across both controls). Washing further the microarray from the non-stringent wash (Fig. 4A) without SDS failed to remove the non-covalent interactions. However, more stringent conditions (higher concentration of SDS or higher temperature) in the stringent wash led to loss of PNA/DNA hybridization. Taken together, these results indicate that we have successfully identified conditions that can discriminate between covalent and non-covalent interaction in a primary screening on the array. The experiment was repeated with MEK2, spiking the library with the four controls (dasatinib, BIM, two ligand known to interact with MEK2;[63] PD-0325901, an allosteric ligand specific for MEK2; canertinib, a covalent ligand that should not react with MEK2). As shown in Fig. 4C, the screen under mild washing condition clearly shows the interaction of dasatinib (position **7-50**), BIM (position **79-75**) and PD-0325901 (position **99-99**) with MEK2. It should be noted that MEK2 has cysteine residues in the vicinity of the nucleotide binding site,[64] but their positions are not suitable to be engaged in a reaction with canertinib. Indeed, the screening result does not show any binding of canertinib despite the presence of the Michael acceptor. Other compounds in the library were identified as potential strong binders. Under the stringent washing conditions with SDS to exclude non-covalent interactions (Fig. 4D), the positive controls that interact non-covalently are now below the detection threshold. Interestingly, there remained high intensity binders suggesting that the library contains compounds that successfully engaged MEK2 in a covalent interaction (See Fig. 5 for highlighted compounds and their structure). Six compounds were resynthesized, replacing the PNA tag with a fluorophore (PEG-Cy3, Fig. 5B) in order to validate their covalent engagement of MEK2. As shown in Fig. 5C, incubation of the compounds with MEK2 for 1h followed by SDS denaturing gel showed that the compounds resulted in a covalent adduct albeit differences in the reaction levels, with compounds **69-7** and **97-7** being most efficient.

To further validate that the compounds reacted with the cysteines in the nucleotide-binding site, competition assays pre-incubating the protein with a known covalent inhibitor targeting the nucleotide binding site (**NW-466**)[65], and with the allosteric inhibitor (PD-0325901) were performed. As shown in Fig. 6A, both competitors inhibited the labeling of MEK2 with **69-7** and **97-7** indicating that the labeling results from an interaction of compounds **69-7** and **97-7** with the nucleotide binding site and that this interaction accelerates the formation of the covalent adduct. Next, we measure the rate of labeling, analyzing the extent of labeling following a denaturing quench of the reaction at different time points (Fig. 5B), showing a half-life of reaction in >10 min in both cases.

Applying the same screening methodology with library II (Fig. 3), we screened ERBB2 using the same four controls and the stringent denaturing wash to reveal the covalent ligands. Again, the non-covalent ligands were not detected but the positive control, canertinib was amongst the strongest hits (Fig. 7). Amongst the other compounds identified, two fragments (**88** and **98**) emerged as the fittest (Fig. 7). These fragments had been used in the development of a covalent inhibitor of EGFR, a kinase with a cysteine at the same position as ERBB2.[66] Three compounds were selected as representative examples and re-synthesized with a fluorophore (Cy3-PEG) in lieu of the PNA (**14-98, 20-98, 32-98**). As shown in Fig. 8, incubation of the compounds with ERBB2 for 60 min followed by SDS denaturing gel clearly showed that three of the compounds labeled the protein in a covalent fashion.

Finally, the selectivity of compound **32-98** lacking a tag was assessed in a displacement assay against a broad panel of kinases (Kinome scan, DiscoverX). As shown in Fig. 9, the compound showed a very good selectivity, with two kinases bearing a cysteine at the same position (ERBB2 and JAK3). In addition, this compound also targeted MEK4 and DAPK3.

Conclusions

In conclusion, we developed a simple procedure to discriminate between covalent and high affinity non-covalent inhibitors using DNA display. Notably, a stringent washing with SDS can be used to remove non-covalent binders, despite their strong target binding (low nM) without compromising the nucleic acid hybridization. Based on the resurging interest in covalent inhibitors and the growing implementation of DNA encoded libraries in the identification of leads, this methodology fills an important gap in the screening of DNA-encoded libraries. While the present screening was performed on microarrays, which facilitates the turnaround in the analysis of results, we anticipate that it can also be used with affinity selection for immobilized targets using next generation sequencing technologies to identify the best inhibitors from a library. The synthetic methodologies developed for the library synthesis add to the repertoire of robust reactions for diversification of PNA-encoded libraries and, demonstrate that cysteine reactive functionalities can be incorporated in encoded libraries. We anticipate that the results presented herein will encourage the discovery of novel covalent inhibitors from DNA or PNA encoded libraries.

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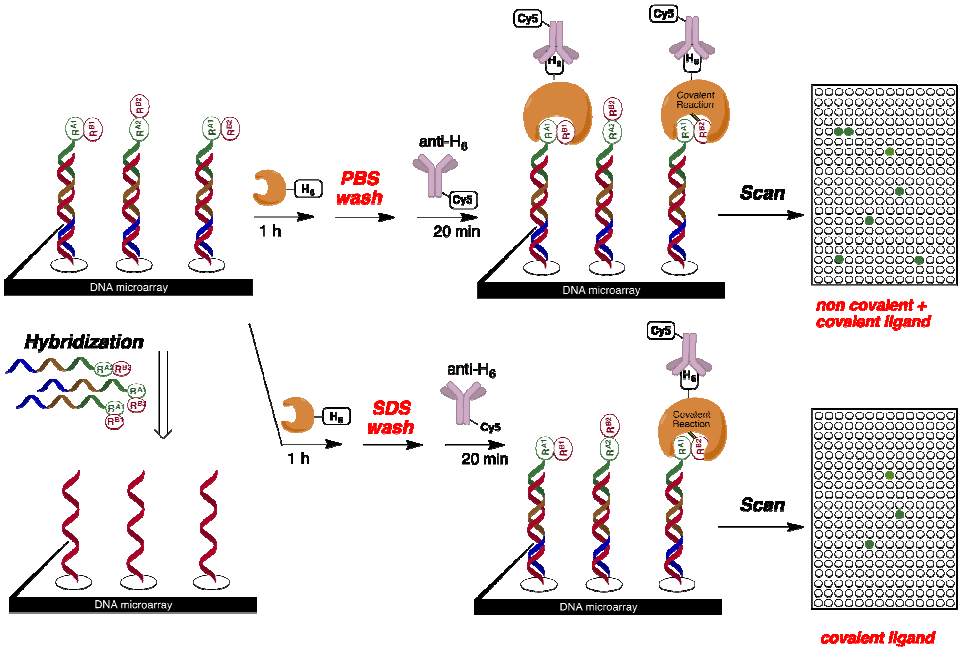

Fig. 1 Discovering covalent binders for irreversible inhibition.

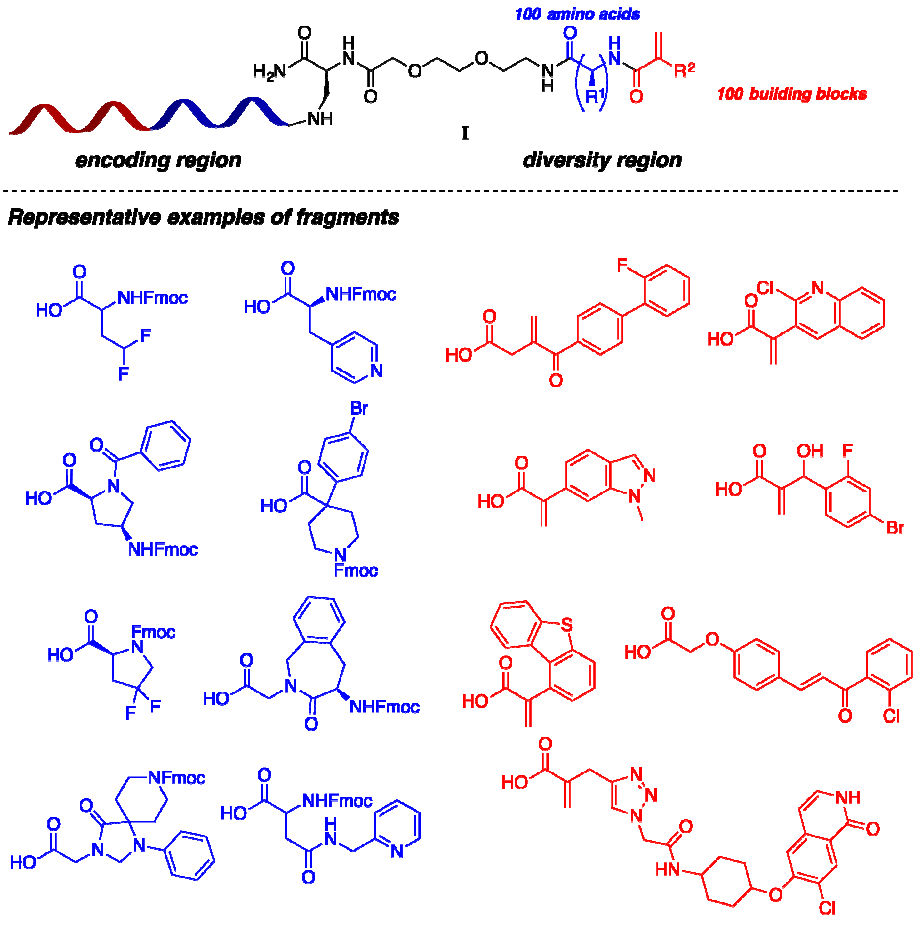

Fig. 2 Design of a generic PNA-encoded library of Michael acceptors, library **I** (entire set of fragments can be found in the supporting information).

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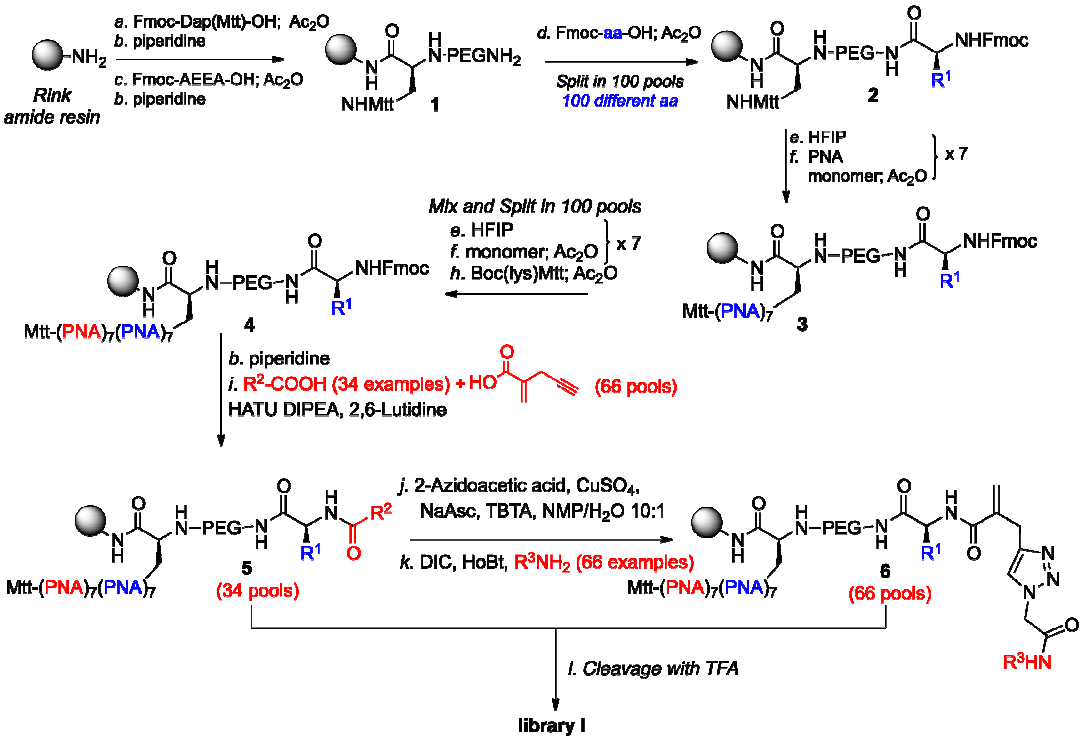

Scheme 1 Synthesis of a generic 10K PNA-encoded library of covalent binders (library **I**).

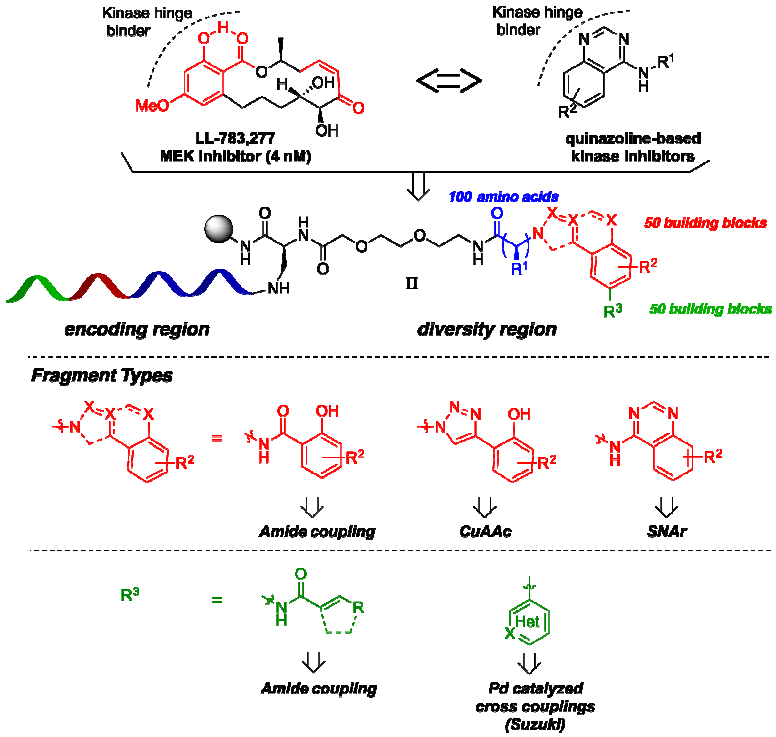

Fig. 3 Design of a kinase targeting PNA-encoded chemical library (library **II**), (full description can be found in the supporting information).

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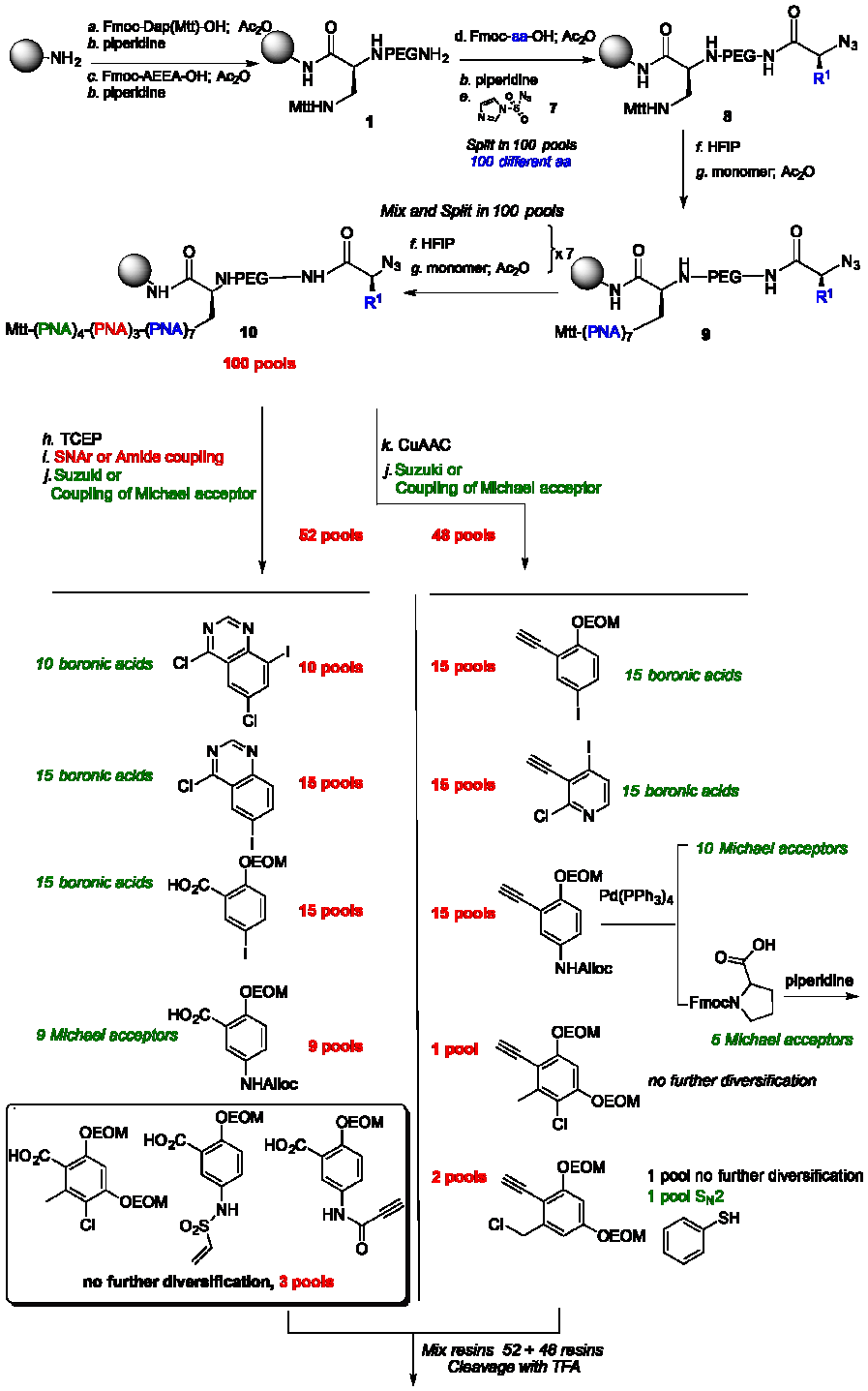

Scheme 2 Synthesis of a kinase targeted PNA-encoded chemical library (library **II**). EOM = Ethoxymethyl.

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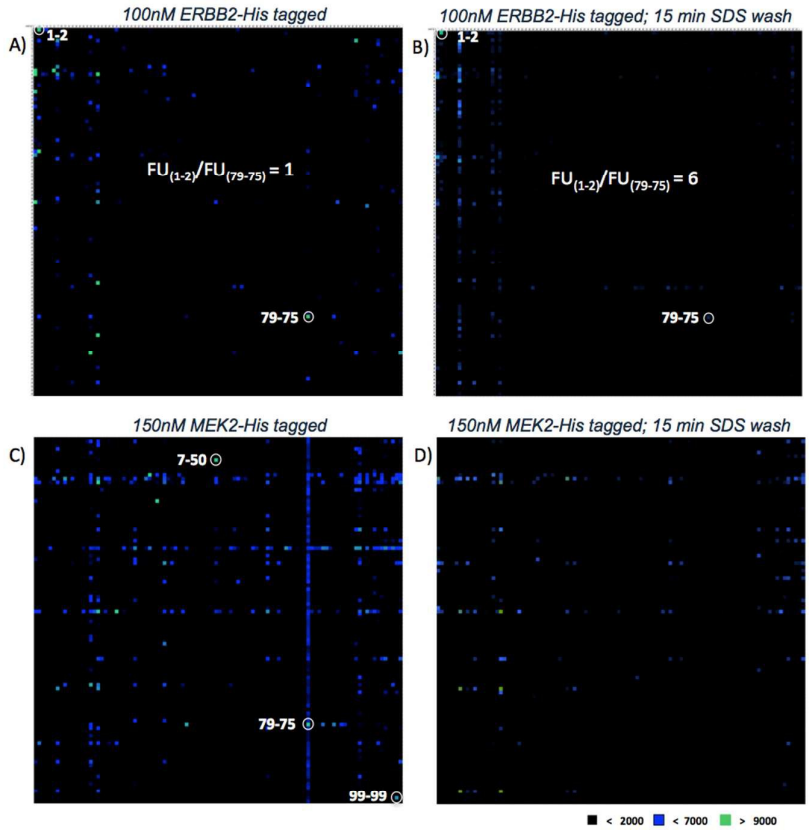

Fig. 4 Microarray screening with generic 10K PNA-encoded library of covalent binders (Library **I**) against A) 100nM **ERBB2**-his tagged with a 15 min PBS wash; B) 100nM **ERBB2**-his tagged with a 15 min SDS wash; C) 150nM **MEK2**-his tagged with a 15 min PBS wash and D) 150nM **MEK2**-his tagged with a 15 min SDS wash. Controls in ERBB2 screens are Canertinib (**1-2**) and BIM (**79-75**). Controls in MEK2 screens are Dasatinib (**7-50**), BIM and PD-0325901 (**99-99**). Controls are Cy3 labeled and the proteins were scanned using a Cy5 labeled antibody.

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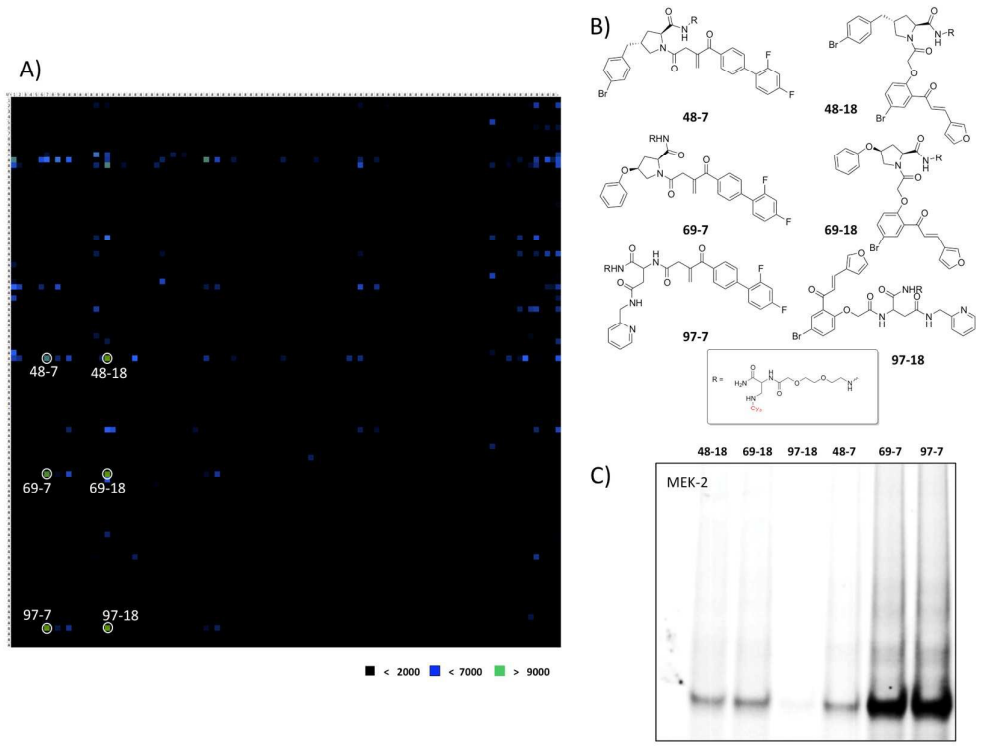

Fig. 5 A) Microarray screening with generic 10K PNA-encoded library of covalent binders (Library **I**) against 150nM **MEK2**-his tagged with a 15 min SDS wash (expended view of Fig 4 D with highlighted hits). Selected compounds to synthesize off-oligo for hit validation. B) Compounds resynthesized off-oligo as Cy3 conjugates. C) Scan of a SDS gel of MEK2-his 500nM labeling with Cy3 conjugates at 2□M in buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 3mM $MgCl_2$, 3mM $MnCl_2$, 0.01% Tween 20, 10 □g of HEK cell extract) for 1 h at RT followed by 5 min denaturation at 95 ^{0}C/SDS.

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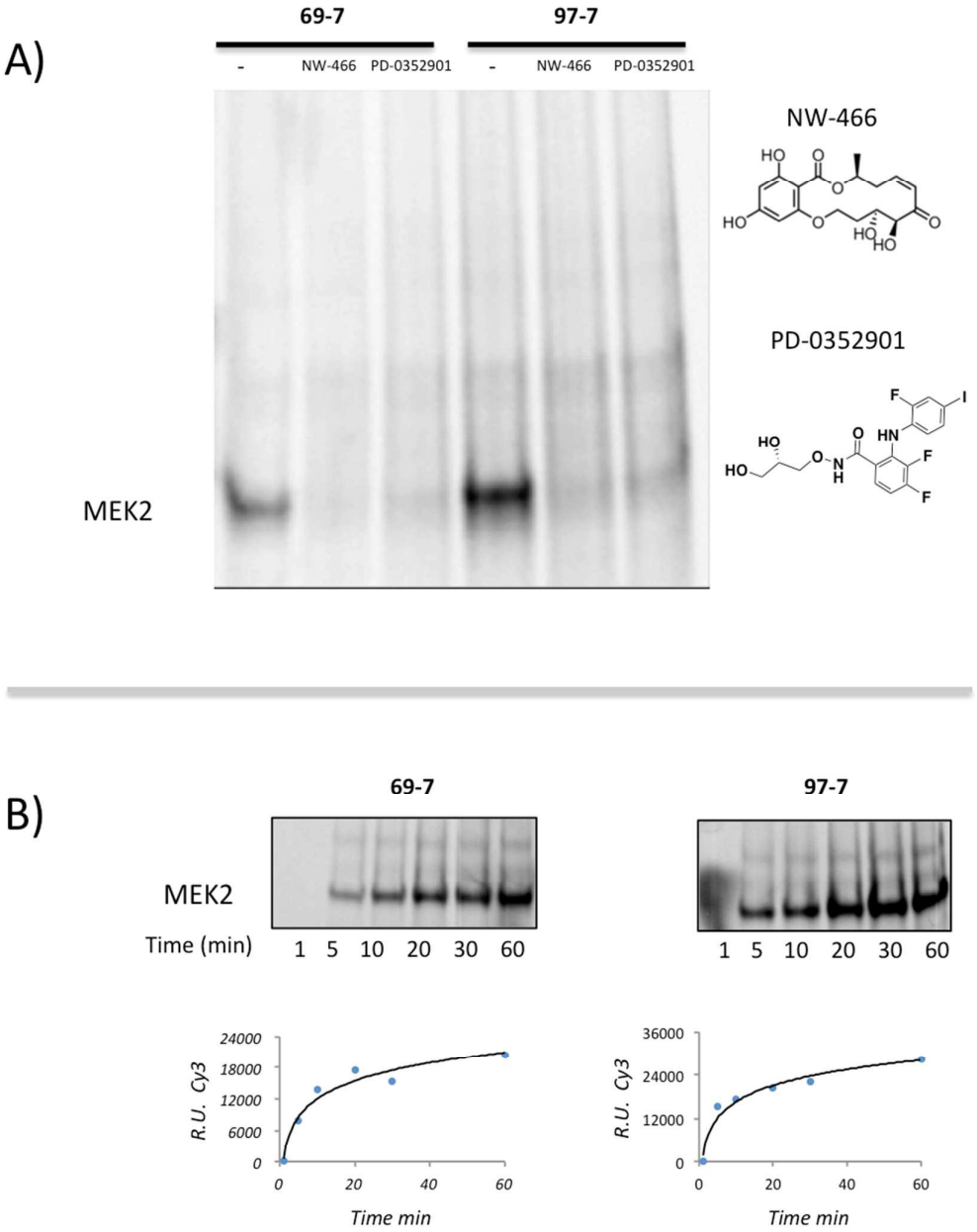

Fig. 6 A) SDS gel scan of the **MEK2**-his 500nM labeling when the enzyme was pre incubated with the corresponding competitor (no competitor, a **hypothemycin** analog **NW-466** or **PD-0325901**) for 30 min at RT followed by incubation with Cy3 conjugates at 2 M in buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 3mM $MgCl_2$, 3mM $MnCl_2$, 0.01% Tween 20, 10 g of HEK cell extract) for 1 h at RT followed by 5 min denaturation at 95 ^{0}C/SDS/10 mM DTT. B) SDS gel scan of **MEK2**-his 500nM labeling with compounds **69-7** and **97-7** Cy3 conjugate at 2 M in buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 3mM $MgCl_2$, 3mM $MnCl_2$, 0.01% Tween 20, 10 g of HEK cell extract) at RT followed by 5 min denaturation at 95 ^{0}C/SDS/10 mM DTT, at different time points to study the kinetics of the covalent binding. The kinetics of labelling was

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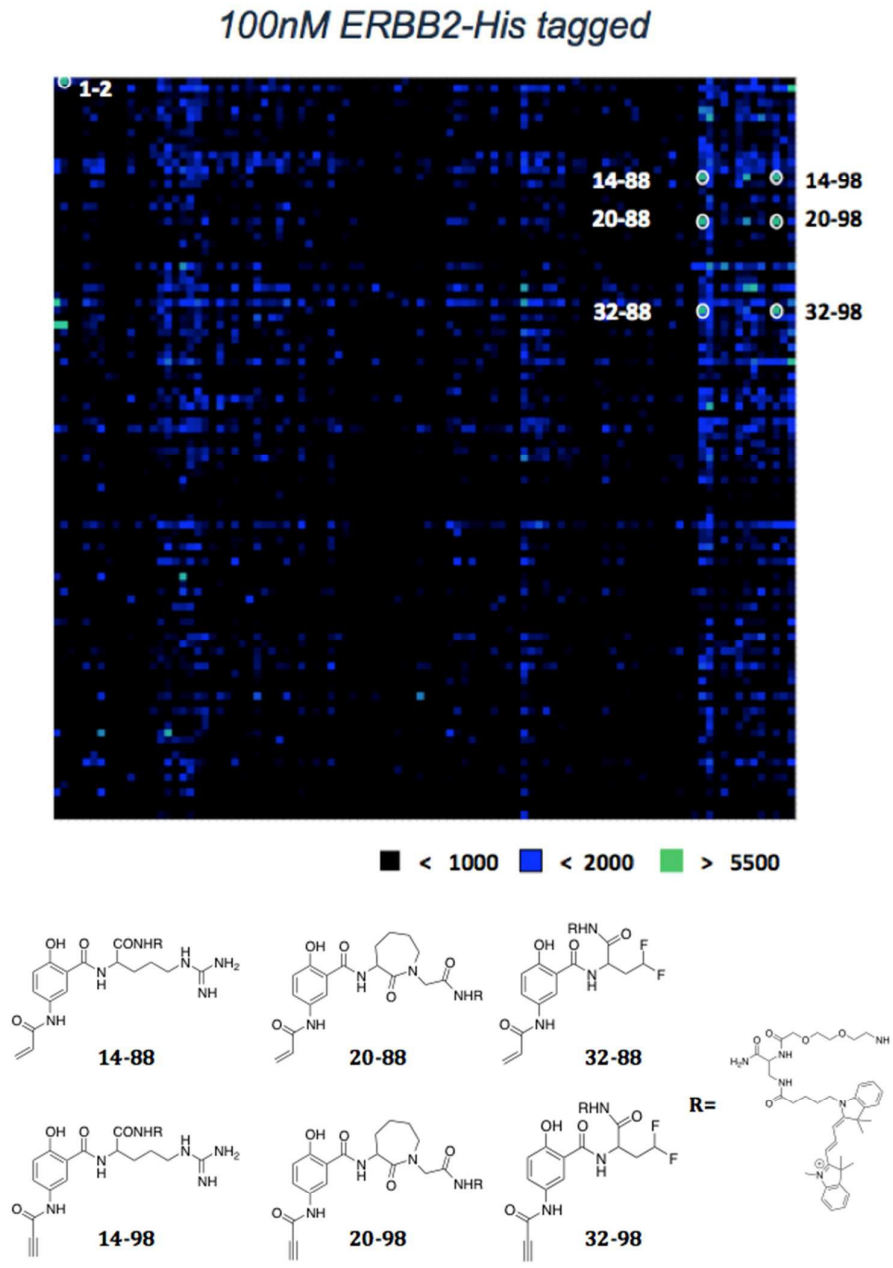

Fig. 7 Microarray screening with kinase targeted PNA-encoded chemical library (***Library II***) against 100nM **ERBB2**-his tagged with a 15 min SDS wash. Controls in the screen is e Canertinib-Cy3 encoded with codons 1-2 spiked in the library. Selected compounds resynthesized off-oligo as Cy3 conjugates.

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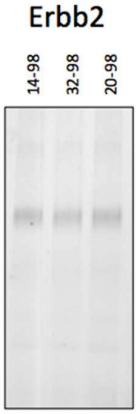
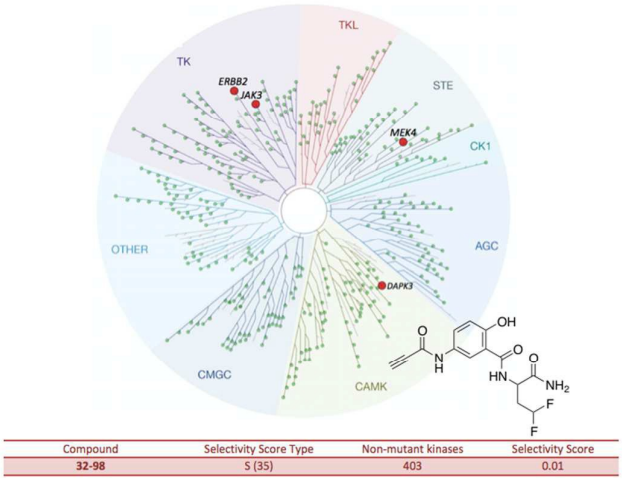


Fig. 8 SDS gel scan of **ERBB2**-his tagged at 500nM labeling with compounds **14-98**, **20-98, 32-98** Cy3 conjugate at 2uM in buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 3mM $MgCl_2$, 3mM $MnCl_2$, 0.01% Tween 20, 10 µg of HEK cell extract) for 1 h at RT followed by 5 min denaturation at 95 ^{0}C/SDS.



Compound	Selectivity Score Type	Non-mutant kinases	Selectivity Score
32-98	S (35)	403	0.01

Fig. 9 KinomeScan™ kinase selectivity profile for 32-98. Unconjugated **32-98** was profiled at a concentration of 1 µM, against a diverse panel of more than 400 kinases by DiscoverX Corporation.

Table of contentn entry: Discriminating between non-covalent and covalent inhibitors with SDS wash in microarray-based screen.