Supplementary Information

Covalent Docking of Large Libraries for the Discovery of Chemical Probes

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Supplementary Results

Supplementary Tables

Supplementary Table 1. Characterization of commercially available and virtual electrophile libraries

| Electrophile                          | Subset^a | N^b | HA^c | Rot^d | MW^e | AlogP^f | Acc^g | Don^h | SMARTS                                      |
|--------------------------------------|----------|-----|------|-------|------|---------|-------|-------|---------------------------------------------|
| Boronic acid                         |          | 22,683 | 20±7.1 | 4.3±2.5 | 283±98.3 | 3.3±1.5 | 3.9±1.4 | 1.8±1.1 | OBO                                         |
| α-ketoamide                          |          | 13,674 | 26.5±3.9 | 5.5±1.7 | 365.5±52.7 | 2.8±1.2 | 3.8±1.2 | 1.2±0.8 | O=[CR0][#6][CR0][=O][N=#6]                  |
| α,β-unsaturated carbonyl             | Lead     | 230,997 | 21.8±2.4 | 4.4±1.6 | 307.8±30.9 | 2.2±1.1 | 3.7±1.4 | 1.0±0.8 | C=CC=O^i                                   |
|                                      | Frag.    | 27,175 | 15.4±2.4 | 2.8±1.4 | 216.5±30.8 | 1.4±1.3 | 2.9±1.2 | 0.7±0.8 |                                            |
| Carbamate                            | Lead     | 76,880 | 21.8±2.4 | 5.2±1.3 | 310.2±31 | 1.5±1.1 | 4.1±1   | 1.4±0.9 | NC(=O)O                                    |
|                                      | Frag.    | 10,468 | 15.3±2  | 3.3±1.2 | 219±26.4  | 1±1.2   | 3.2±1   | 1.1±0.8 | [CX3H1](=O)[#6]                            |
| Aldehyde                             | Lead     | 21,897 | 20.4±3.6 | 4.8±1.7 | 288.4±44.7 | 2.2±1.1 | 3.6±1.3 | 0.7±0.8 | [Br,Cl,I][CX4;CH,CH2]                      |
| Alkyl halides                        | Lead     | 17,715 | 16.6±3.5 | 3.7±1.7 | 275.2±43.3 | 2.2±1   | 2.6±1.4 | 0.7±0.8 |                                            |
| Epoxide                              | Lead     | 8,306  | 22.5±7.9 | 4±3.1  | 317.4±108.5 | 2.3±1.7 | 3.9±1.9 | 0.6±0.9 | C1CO1                                      |
|                                      | Frag.    | 11,525 | 18.5±2.8 | 3.7±1.3 | 258.5±37.1 | 1.1±1.1 | 3.8±1.2 | 1.5±0.8 | See Supp. Fig. 9                           |
| Aldehyde based cyanoacrylamides      | Frag.    | 8,306  | 22.5±7.9 | 4±3.1  | 317.4±108.5 | 2.3±1.7 | 3.9±1.9 | 0.6±0.9 | C1CO1                                      |
|                                      |          | 11,525 | 18.5±2.8 | 3.7±1.3 | 258.5±37.1 | 1.1±1.1 | 3.8±1.2 | 1.5±0.8 | See Supp. Fig. 9                           |
| Suzuki based cyanoacrylamides        | Frag.    | 225,868 | 24.6±2.2 | 4.2±1   | 337.5±29.2 | 1.9±1   | 4.4±1.1 | 1.7±0.7 | See Supp. Fig. 11                          |
|                                      |          |        |        |        |        |         |         |       |                                             |

^a Subset of ZINC^1 used as an additional filter:
Lead-like: 250 < molecular weight < 350; xLogP ≤ 3.5; rotatable bonds ≤ 7
Fragments: molecular weight < 250; xLogP ≤ 3.5; rotatable bonds ≤ 5
Fragments-now: same as fragments, limited to ‘in-stock’ compounds.
^b Number of molecules in the library
Average (± standard deviation): ^c number of heavy atoms; ^d number of rotatable bonds; ^e molecular weight; ^f calculated logP (as implemented in Pipeline Pilot^2)
^g number of hydrogen bond acceptors; ^h number of hydrogen bond donors.
^i Note that this expression might capture less reactive moieties such as β-disubstituted enones.
^j We did not include fluorine as most alkyl fluorides are non-reactive. Note however that fluoromethylketones are also excluded by this SMARTS pattern.
Supplementary Table 2. Comparison of covalent modeling of β-lactams with other docking software.

|            | DOCKovalent | CovalentDock | AutoDock | GOLD |
|------------|-------------|--------------|----------|------|
| Median (Å)\(^a\) | 2.36\(^b\)  |              |          |      |
| Average (Å)\(^a\) | 2.87        | 3.4\(^c\)   | 3.5\(^c\) | 4.0\(^c\) |

\(^a\)The median/average Root Mean Square Deviation (RMSD) obtained by different covalent docking software over n=61 β-lactam adducts (Supplementary Table 3; \(^3\)).

\(^b\)The difference between the average and median performance is due to a few outliers with high RMSD values (Supplementary Table 3). A clear tendency was observed for high RMSDs for ligands with more than 11 rotatable bonds.

\(^c\)Values are extracted from ref. [1] figure 10 for the top 1 prediction (as was used by DOCKovalent) note that these averages include 13 more non β-lactam structures.
Supplementary Table 3. β-lactam pose recapitulation benchmark*

| PDB   | #rot. | RMSD (Å) | PDB   | #rot. | RMSD (Å) | PDB   | #rot. | RMSD (Å) |
|-------|-------|----------|-------|-------|----------|-------|-------|----------|
| 3BEB  | 6     | 2.12     | 2EX9  | 8     | 0.88     | 1FCO  | 11    | 3.43     |
| 1IYP  | 7     | 1.94     | 1YMX  | 8     | 1.42     | 3N8S  | 11    | 2.21     |
| 2JBF  | 7     | 1.51     | 2ZC5  | 8     | 7.03     | 3OCN  | 12    | 1.73     |
| 2J8Y  | 7     | 0.96     | 3MZE  | 8     | 1.65     | 1BT5  | 12    | 4.93     |
| 1GHM  | 7     | 0.96     | 3ITA  | 8     | 2.69     | 3MZF  | 12    | 2.13     |
| 3LY4  | 7     | 2.27     | 2ZC3  | 8     | 2.36     | 3PBO  | 12    | 4.49     |
| 1GHP  | 7     | 2.40     | 1FCN  | 8     | 2.66     | 2ZQD  | 12    | 4.59     |
| 2ZD8  | 7     | 1.22     | 1B12  | 8     | 1.63     | 1PWD  | 12    | 2.36     |
| 1IYQ  | 7     | 1.56     | 3OCL  | 9     | 2.78     | 2Z2M  | 12    | 1.86     |
| 2EX8  | 7     | 0.96     | 2ZQA  | 9     | 1.66     | 3M6H  | 12    | 7.85     |
| 3MZD  | 7     | 4.60     | 2ZC6  | 9     | 1.33     | 1LL5  | 12    | 5.15     |
| 2ZQ9  | 7     | 3.23     | 2ZC4  | 9     | 1.24     | 3M6B  | 12    | 6.74     |
| 2EXA  | 7     | 2.56     | 1CEF  | 9     | 3.57     | 3IQA  | 12    | 4.70     |
| 1KVM  | 7     | 2.15     | 1LLB  | 9     | 6.03     | 2VGJ  | 13    | 4.25     |
| 1W8Y  | 7     | 3.49     | 1LL9  | 9     | 1.18     | 2ZQC  | 13    | 1.90     |
| 1CEG  | 7     | 1.04     | 3DWZ  | 9     | 2.11     | 1FR6  | 13    | 6.44     |
| 1FCM  | 7     | 2.46     | 2CSW  | 9     | 2.69     | 1PW8  | 14    | 3.57     |
| 1QMF  | 8     | 2.68     | 2XD1  | 10    | 3.97     | 1PWG  | 15    | 4.07     |
| 3A3I  | 8     | 1.99     | 3A3F  | 10    | 3.32     | 3EXB  | 17    | 3.88     |
| 2EX6  | 8     | 1.38     | 3A3E  | 10    | 1.81     | 15Q   | 11    | 3.71     |

a Number of rotatable bonds in the covalent adduct.
b PDBs in italics indicate cases where different protonation states were available. The lower RMSD is reported in these cases.
* Note that the original benchmark reported in 3 contained two additional cases 3BEC and 3KGO, however these had non-realistic covalent bond angles and were excluded from the analysis.
Supplementary Table 4. DOCKovalent retrospective virtual screen performance

| Target | PDB  | Electrophile | Type<sup>a</sup> | Lib. Size<sup>b</sup> | Ligands<sup>b</sup> | AUC<sup>d</sup> | logAUC<sup>e</sup> |
|--------|------|--------------|-------------------|-----------------------|---------------------|-------------|------------------|
| EGFR   | 4G5J | α,β-unsaturated carbonyl | Product | 215,000<sup>c</sup> | 50 | 85.2 | 27.5 |
| FAAH   | 3LJ7 | Boronic Acid | Product | 11,000 | 142 | 84.3 | 25.1 |
|        |      | Carbamate   | HEI   | 71,000<sup>c</sup> | 61 | 77.8 | 13.5 |
| AChE   | 4EY6 | Carbamate   | HEI   | 72,000<sup>c</sup> | 232 | 74.8 | 17.0 |
| NS3    | 1RTL | α-Ketoamide | HEI   | 13,500 | 93 | 16.5 | -10.7 |

<sup>a</sup> Covalent ligands were docked either in their product form, or in their High Energy Intermediate (HEI) form (Supplementary Fig. 1).

<sup>b</sup> We report the number of molecules (both ligands and decoys) for which a non-clashing pose was found. E.g. for EGFR poses were found for 50 known inhibitors and 215,000 decoys containing an α,β-unsaturated carbonyl group.

<sup>c</sup> ZINC was filtered for molecules containing the electrophiles. To limit the docking library size for the α/β-unsaturated carbonyl and carbamate libraries we included only lead-like molecules (250 ≤ molecular weight ≤ 350 ; xLogP ≤ 3.5 ; Number of rotatable bonds ≤ 7).

<sup>d</sup> Area Under ROC curve (AUC). 100% corresponds to perfect ranking. 50% corresponds to random ranking.

<sup>e</sup> Adjusted logAUC is a measure for early enrichment<sup>4</sup>, and random ranking corresponds to a logAUC of 0.
Supplementary Table 5. RMSDs for AmpC/Boronic acid pose recapitulation benchmark

| PDB   | RMSD(Å) | PDB   | RMSD(Å) | PDB   | RMSD(Å) |
|-------|---------|-------|---------|-------|---------|
| 1KDS  | 0.43    | 1MXO  | 1.11    | 4E3O  | 2.43    |
| 4E3M  | 0.62    | 2RCX  | 1.15    | 1IEM  | 3.05    |
| 4E3N  | 0.65    | 1C3B  | 1.19    | 1GA9  | 3.37    |
| 1MY8  | 0.67    | 3O86  | 1.72    | 1FSW  | 3.49    |
| 1KDW  | 0.69    | 4E3L  | 1.73    | 3BM6  | 3.53    |
| 4E3J  | 0.74    | 3O88  | 1.93    | 3BLS  | 5.47<sup>a</sup> |
| 1KE0  | 0.91    | 3O87  | 1.97    | 4LV0  | 0.46<sup>b</sup> |
| 4E3K  | 0.97    | 2I72  | 2.19    | 4E3I  | 6.66    |

<sup>a</sup> RMSD to previously deposited incorrect pose. See results section on MAPB.

<sup>b</sup> RMSD to newly deposited MAPB structure.
**Supplementary Table 6. Specificity of new boronic acids for AmpC**

| Compound | 2   | 3   | 5   | 7   |
|----------|-----|-----|-----|-----|
| AmpC     | 0.18| 0.04| 0.6 | 0.01|
| Trypsin  | >5mM| 138 | >5mM| 1306|
| Elastase | 248 | 3331| 382 | 2882|
| α-CT     | 167 | 0.3 | 1010| 99  |

| Specificity<sup>b</sup> | Trypsin | >27,777 | 3450  | >8,333 | 130600 |
|-------------------------|---------|---------|-------|--------|--------|
| Elastase                | 1378    | 83275   | 637   | 288200 |
| α-CT                    | 928     | 8       | 1683  | 9900   |

<sup>a</sup> $K_i$ is calculated based on single point measurement, based on literature $K_m$ values of respective protease substrates (see Methods).

<sup>b</sup> Specificity is calculated as $K_i$ Protease/$K_i$ AmpC.
**Supplementary Table 7. Lack of activity of predicted AmpC non-binders**

| Compound | Dock Rank | $K_i$ [μM] | Ligand Eff. $^a$ |
|----------|-----------|------------|-----------------|
| 14       | 10694     | 3.21$^b$   | 0.43            |
| 15       | 10706     | N/A$^c$    | N/A             |
| 16       | 10819     | N/A        | N/A             |
| 17       | 10835     | N/A        | N/A             |
| 18       | 10982     | N/A        | N/A             |

$^a$ Ligand efficiency based on the calculated $K_i$

$^b$ IC$_{50}$ was calculated based on a full dose response curve (Supplementary Fig. 15)

$^c$ N/A: < 10% inhibition at 10 μM
Supplementary Table 8. Antibacterial activity of new AmpC inhibitors in the absence of cefotaxime

| Strain \(^a\)       | MIC (µg/ml) | 2 | 3 | 5 | 7 |
|----------------------|-------------|---|---|---|---|
| Citrobacter freundii | > 2048      | > 2048 | 2048 | 2048 |   |
| Enterobacter cloacae | > 2048      | > 2048 | 1024 | 1024 |   |
| Enterobacter aerogenes| > 2048      | > 2048 | 2048 | 1024 |   |
| Escherichia coli Hcase| > 2048     | > 2048 | 1024 | 2048 |   |
| Escherichia coli Hcase| > 2048     | > 2048 | 1024 | 1024 |   |
| Escherichia coli TEM3 | > 2048      | > 2048 | 1024 | 1024 |   |
| Escherichia coli CTXM14| > 2048      | > 2048 | 1024 | 2048 |   |

\(^a\) clinical isolates previously shown to be resistant to third generation cephalosporins.
Supplementary Table 9. Docking ranks of Suzuki library combinations

| # hits<sup>a</sup> | PDB<sup>b</sup> | Rank | PDB | Rank | PDB | Rank | PDB | Rank | Best linker<sup>c</sup> |
|-------------------|-----------------|------|-----|------|-----|------|-----|------|-------------------------|
| 2                 | 3lxl            | 636  | 3lxk| 639  | 3lxk| 948  | 3lxk| 16   |                         |
| 4                 | 3lxk            | 425  | 4hvg| 183  | 4hvg| 5562 | 4hvg| 183  |                         |
| 2                 | 3lxl            | 525  | 4hvg| 1406 | 4hvh| 6906 | 4hvg| 66   |                         |
| 1<sup>d</sup>     | 3lxl            | 290  | 4hvg| 5700 | 4hvg| 3056 | 3lxl| 290  |                         |
| 7                 | 3pjc            | 1    | 4hvd| 3    | 3lxl| 10   | 3pjc| 1    |                         |
| 5                 | 3pjc            | 121  | 3pjc| 164  | 3pjc| 488  | 4hvi| 22   |                         |
| 4                 | 3pjc            | 1235 | 3pjc| 2003 | 4hvg| 98   | 3lxl| 73   |                         |
| 8                 | 3lxl            | 505  | 3lxk| 411  | 4hvg| 149  | 3lxk| 6    |                         |
a Number of PDB templates for which this scaffold ranked with any linker in the top 500. b PDB template for which this combination of scaffold and linker scored best. c The best rank of this scaffold with any of the 50 boronic acid aldehyde linkers; the linker that achieved this rank is depicted in the right-most column. d The only candidate chosen using a native Cys909 rotamer (+60), all other compounds were selected based on docking to an alternative Cys rotamer (–60).
**Supplementary Table 10. Data collection and refinement statistics**

|                     | AmpC/MAPB (4LV0) | AmpC/3 (4LV1) | AmpC/7 (4LV2) | AmpC/14 (4LV3) | RSK2 T493M/24 (4M8T) |
|---------------------|------------------|---------------|---------------|----------------|----------------------|
| **Data collection** |                  |               |               |                |                      |
| Space group         | C2               | C2            | C2            | C2             | P4₁2₁2               |
| Cell dimensions     |                  |               |               |                |                      |
| $a$, $b$, $c$ (Å)   | 118.46, 77.46, 97.51 | 118.58, 77.54, 97.64 | 117.67, 78.14, 97.25 | 117.67, 78.14, 97.25 | 46.99, 46.99          |
| $\alpha$, $\beta$, $\gamma$ (°) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å)      | 50-1.65          | 50-1.74       | 50-1.65       | 50-1.42        | 46.38 – 3.0          |
| $R_{merge}$         | 3.1 (51.9)       | 4.1 (53.1)    | 5.6 (75.3)    | 3.5 (42.1)     | 18.5 (20.9)          |
| $I/\sigma I$        | 20.6 (2.1)       | 20.6 (2.4)    | 14.8 (2.1)    | 21.7 (3.5)     | 13.1 (6.0)           |
| Completeness (%)    | 96.5 (94.1)      | 99.7 (99.8)   | 99.5 (96.9)   | 96.5 (93.9)    | 98.7 (99.9)          |
| Redundancy          | 2.6 (2.5)        | 3.7 (3.7)     | 4.5 (4.15)    | 3.9 (3.6)      | 23.4 (26.1)          |
| **Refinement**      |                  |               |               |                |                      |
| Resolution (Å)      | 45.67-1.65 (1.675-1.65) | 45.55-1.74 (1.769-1.74) | 45.58-1.65 (1.673-1.65) | 44.18-1.42 (1.436-1.42) | 46.39 – 3.0 (3.78 – 3.00) |
| No. reflections     | 91645            | 81114         | 94599         | 144017         | 7265                 |
| $R_{work}/R_{free}$ | 16.4 (25.5) / 19.4 (29.7) | 17.0 (25.4) / 19.5 (28.7) | 17.2 (29.7) / 19.2 (29.8) | 17.8 (23.9) / 19.2 (25.1) | 25.3 (29.8) / 31.3 (38.0) |
| No. atoms           |                  |               |               |                |                      |
| Protein             | 5558             | 5542          | 5538          | 5507           | 2370                 |
| Ligand/ion          | 25               | 70            | 55            | 39             | 20                   |
| Water               | 802              | 495           | 428           | 699            | 0                    |
| $B$-factors         |                  |               |               |                |                      |
| Protein             | 20.8             | 19.9          | 23.6          | 25.2           | 59.5                 |
| Ligand/ion          | 20.3             | 21.2          | 29.6          | 26.5           | 55.3                 |
| Water               | 29.8             | 26.0          | 30.2          | 35.8           | 55.1                 |
| R.m.s. deviations   | Bond lengths (Å) | 0.014         | 0.014         | 0.012          | 0.011                |
|                      | Bond angles (°)  | 1.51          | 1.48          | 1.46           | 0.91                 |

* All datasets were collected from single crystals. The highest-resolution shell is shown in parentheses.
Supplementary Table 11. Experimental details for JAK3 kinase selectivity assays

| Kinase | Vendor # | [enzyme] nM | [ATP] µM | Incubation (hours) |
|--------|----------|-------------|----------|-------------------|
| JAK1   | Invitrogen-PV4774-877058D | 0.5 | 70 | 2 |
| JAK2   | Invitrogen-PV4210-784633G | 0.2 | 12 | 2 |
| JAK3   | Invitrogen-PV3855-1026644 | 0.5 | 2 | 2 |
| TYK2   | Invitrogen-PV4790-884908A | 1.0 | 35 | 2 |
| BLK    | BPS-40401-111102 | 0.1 | 20 | 3 |
| BMX    | BPS-40402-110711 | 0.5 | 75 | 3 |
| BTK    | BPS-40405-130315-GC2 | 0.7 | 16 | 3 |
| EGFR   | BPS-40187-131015-G2 | 0.4 | 3 | 3 |
| ERB-B2 | BPS-40230-110913-5 | 2.0 | 50 | 3 |
| ERB-B4 | CARNA-08-118 -08CBS-0652 | 0.8 | 15 | 3 |
| TEC    | CARNA-08-182-10CBS-0017 | 1.0 | 50 | 3 |
| ITK    | CARNA-08-181-10CBS-1259 | 0.2 | 10 | 4 |
| TXK    | INVITROGEN-PV5860-750657B | 0.5 | 100 | 3 |
Supplementary Figures

**Supplementary Figure 1. Electrophiles used in this study.**
Structures of electrophiles available for docking, from top to bottom: α,β-unsaturated carbonyl, aldehyde, boronic acid, cyanoacrylamide, alkyl halide, carbamate, α-ketoamide and epoxide. The electrophilic atom is indicated in red. The high-energy intermediate (center) or product (right) form for each electrophile is depicted as a covalent adduct to a protein nucleophile (cysteine or serine). Note that in some cases a new chiral center is formed upon reaction with the target nucleophile.
Supplementary Figure 2. Sampling parameters of DOCKovalent

Schematic of an electrophilic ligand (L₁-L₄) covalently attached to a Cys residue (for illustration). The dihedral angles φ₁ (Cα-Cβ-Sγ-L₁) and φ₂ (Cβ-Sγ-L₁-L₂) are exhaustively sampled in steps of 20°. The covalent bond length ‘d’ and bond angles ‘a’ (Cβ-Sγ-L₁) and ‘b’ (Cβ-Sγ-L₄) are set to user-specified ideal values, which are electrophile-specific and sampled within a range of these values (range and sampling steps are also user specified). See Methods section for the values used in this study.
Supplementary Figure 3. Retrospective pose recapitulation of boronic acids binding to AmpC.
DOCKvalent pose predictions (magenta) of boronic acid binding to AmpC (white) closely match the crystal pose (yellow) of PDBs: a. 1KDS (0.43 Å) b. 4E3M (0.62 Å) c. 1MY8 (0.67 Å). See Supplementary Table 5 for RMSD values of the entire benchmark.
Supplementary Figure 4. Docking predictions of new boronic acid AmpC inhibitors.

All boronic acids docked to AmpC with a canonical binding mode, where the boronic acid occupies the oxyanion hole consisting of the backbone amides of Ala318 and Ser64, and accepts a hydrogen bond from Tyr150. a, b. Pyridyl boronic acids 2 and 5, respectively, are predicted to hydrogen bond to Asn152 and Gln120 via the pyridine nitrogen and the ether oxygen. c. Indole boronic acid 4 is predicted to stack against Tyr221 and form a hydrogen bond with the backbone carbonyl of Ala318. The hydrophobic benzyl ether is predicted to project into a hydrophobic pocket created by Thr319 and Val211. d. Methoxypyridine 6, which scored poorly in the docking run, was selected for testing because several high-ranking derivatives of this compound were not available for purchase. See Table 1 for the docking ranks, $K_i$ and ligand efficiencies of these inhibitors.
Supplementary Figure 5. AmpC inhibition by analogs of compound 3.
Structures of each boronic acid analog of 3, and the percent inhibition of AmpC at 1 μM inhibitor. This SAR series shows a preference for substitution at the meta position of the phenyl or pyrimidine substituent.
Supplementary Figure 6. New AmpC boronic acid inhibitors are selective against the yeast proteasome. 

**a.** Proteasome activity in the presence of compounds 2,3,5 and 7 at a concentration of 100 μM or the proteasome activator complex PA26 at a concentration of 5 nM was monitored over time. 

**b.** The substrate hydrolysis rates do not show significant inhibition of the proteasome by the compounds. Rates were determined for the last 10 minutes of the experiment.
Supplementary Figure 7. AmpC adopts a unique conformation to bind compound 14. Binding of compound 14 to AmpC (red) induces a unique conformation of loop 117-120, with Leu119 adopting a rotamer that is not observed in 23 published complex structures of AmpC with boronic acids (green).
Supplementary Figure 8. Retrospective docking of cyanoacrylamides to RSK2. DOCKovalent predictions (magenta) accurately recapitulate the crystallographic poses (yellow) of cyanoacrylamide inhibitors of RSK2.  a. Recapitulation of ligand binding in PDB: 4D9T with an RMSD of 0.66 Å overall (0.48 Å over the scaffold alone). b. Recapitulation of ligand binding in PDB: 4JG8 with an RMSD of 1.52 Å overall (0.91 Å for the scaffold alone).
Supplementary Figure 9. Cyanoacrylamides can be synthesized from aldehydes by Knoevenagel condensation.
Supplementary Figure 10. Crystal structure of compound 24 covalently bound to T493M RSK2. The co-crystal structure of compound 24 (yellow) bound to T493M RSK2 (white) was determined at 3.0 Å resolution. Even at this modest resolution, the electron density (Fo-Fc omit map in green) allowed unambiguous modeling of the phenylpyrazole fragment and the covalent bond to Cys436.
Supplementary Figure 11. A combinatorial library of cyanoacrylamides can be synthesized in two steps: 1. Suzuki-Miyaura cross-coupling of an aldehyde containing boronic acid followed by 2. Knoevenagel condensation to form the final cyanoacrylamide. This scheme is exemplified by fragments that can be used to synthesize compound 27. The in silico virtual library based on this scheme combined 50 boronic acids with 4,397 aryl bromide fragments to form a final library of 219,850 cyanoacrylamides.
Supplementary Figure 12. Docking pose predictions for compounds 31 and 33 in complex with JAK3. a. compound 31 packs its meta-phenyl linker against Leu828, while placing an indazole moiety to form hydrogen bonds with the hinge backbones of Leu905 and Glu903 b. compound 33 forms the same hydrogen bonds to the hinge via an alternative placement of an imidazo[4,5-b]pyridine.
Supplementary Figure 13. **Compound 31 is selective against most kinases that contain a cysteine equivalent to JAK3 Cys909.** Dose-response curves for compound 31 against the nine additional human kinases containing an homologous cysteine to JAK3.
Supplementary Figure 14. Newly discovered covalent inhibitors are reversible. **a.** Compounds 3 and 7 were tested for inhibition at about 5 x IC$_{50}$ concentration with no incubation time (starting the reaction by adding AmpC) and showed ~80% inhibition. When incubated at this concentration for 5 minutes and then diluted 10x the inhibition of ~30% reflected the new diluted concentration and not the high incubation concentration – indicating these compounds are rapidly reversible. **b.** Compounds 24 and 31 were incubated with RSK2 (0.5 hour) and JAK3 (1 hour) respectively at ~IC$_{90}$ concentration. They were then diluted 20x into reaction buffers containing high ATP concentration (100 μM) and either the same concentration of the inhibitor (left columns) or no inhibitor (right columns). The partial retention of inhibition seen for 24 after dilution indicates that it has a relatively slow off-rate, as was previously reported for RSK2 cyanoacrylamide inhibitors. Nevertheless, both compounds are reversible.
Supplementary Figure 15. Raw images of cell-based autophosphorylation assays. Cropped gel images are shown in Figure 3.

| [27] (uM) | 0 | 1 | 0.1 |
|-----------|---|---|-----|
| PMA       | + | - | +   |

| [inhibitor] (μM) | 0 | 20 | 5 | 0.5 | 20 | 5 | 0.5 |
|------------------|---|----|---|-----|----|---|-----|
| PMA              | + | +  | + | +   | +  | + | +   |

- pS380 RSK2
- HA
- pS376 MSK1
- HA
- pS376 MSK1 CV
- HA

6Sk7

PMA + - + + +
Supplementary Figure 16. IC<sub>50</sub> curves for boronic acids 2, 3, 5, 7 and 14

IC<sub>50</sub> values were determined by non-linear regression and K<sub>i</sub> values were calculated using GraphPad software assuming competitive inhibition. The substrate (CENTA) concentration was 50 μM (in a,c,e) or 100 μM (b). All Hill-slopes were approximately –1, indicating a 1:1 inhibitor stoichiometry.
Supplementary Figure 17. IC\textsubscript{50} curves for cyanoacrylamides 19 - 26
IC\textsubscript{50} values were determined by non-linear regression using GraphPad software assuming competitive inhibition.
**Supplementary Notes**

**Supplementary Note 1. Retrospective assessment of covalent docking.** We tested method's ability to find known covalent ligands and geometries in retrospective calculations. In a pose recapitulation benchmark of 61 irreversibly bound β-lactams, the ligand structures predicted by DOCKovalent corresponded to the experimental structures with a median RMSD of 2.36 Å (Supplementary Tables 2,3), compared to an average 3.4 Å in the earlier study. This control calculation supports at least the ability of the method to recapitulate known geometries.

In addition to predicting geometries, a virtual screening method seeks to discover new ligands. A widely-used control for its ability to do so is to dock libraries composed of annotated ligands combined with decoy molecules that resemble the ligands but are not expected to bind. We therefore compiled libraries of known covalent inhibitors for each of the following five targets: epidermal growth factor receptor kinase (EGFR), fatty acid amide hydrolase (FAAH), acetylcholinesterase (AChE), and HCV protease NS3. The different inhibitors were driven by different electrophiles, including Michael acceptors, carbamates, boronic acids and α-ketoamides (Supplementary Table 4, Supplementary Fig. 1). To these libraries we added all purchasable molecules containing the same electrophiles to serve as decoys (Supplementary Table 4). While these “decoy” libraries likely contain genuine covalent inhibitors for these targets, we expected known binders to rank at the top of the hit list.

In four of the five covalent virtual screens, we observed substantial early enrichment of the annotated inhibitors versus the decoy molecules (area under the curve (AUC) of the Receiver Operating Characteristic (ROC) curve > 75%). Thus the adjusted logAUC, a metric that emphasizes early enrichment, was greater than 13.5; an adjusted logAUC of 0 corresponds to random ranking. For NS3, the method performs poorly, likely due to the large number of rotatable bonds in known NS3 inhibitors, which on average had 17.3 rotatable bonds versus between 4.3 and 7.1 rotatable bonds for inhibitors of the other targets.
Supplementary Note 2. Synthetic Chemistry. All purchased chemicals were used as received without further purification. Solvents were dried by passage through columns (either alumina or activated molecular sieves) on a Glass Contour solvent system. NMR spectra were obtained on a Varian Inova 400 MHz spectrometer and referenced to the residual solvent peak. LC-MS analysis was performed on a Waters Acquity LCT UPLC equipped with a TUV detector (monitored at 254 nm) and a Waters Acquity UPLC 1.7 µm C-18 column, eluting at 0.6 mL/min with a 2.5 or 5 minute water:MeCN (with 0.1% formic acid) gradient method.

3-[(1H-1,2,4-triazol-1-yl)methyl]thiophen-2-yl]-2-cyanoacrylamide (19)
4-[(1H-1,2,4-triazol-1-yl)methyl]thiophene-2-carbaldehyde (25 mg, 0.129 mmol) was dissolved in THF (0.5 mL) in a vial with a stirbar, to which was added 2-cyanoacetamide (11 mg, 0.129 mmol) and piperidine (12 µL, 0.129 mmol). The reaction was stirred for 7 hours. The precipitate was collected by filtration and dried in vacuo to afford cyanoacrylamide 19 (17 mg, 51%) as a tan solid. 1H NMR (400 MHz, DMSO) δ 8.64 (s, 1H), 8.32 (s, 1H), 8.01 (s, 1H), 7.94 (s, 1H), 7.79 (br s, 1H), 7.74 (s, 1H), 7.69 (br s, 1H, overlaps peak at 7.74), 5.47 (s, 2H). 13C NMR (125 MHz, DMSO) δ 162.52, 151.82, 144.18, 143.09, 137.99, 137.00, 136.31, 132.46, 116.46, 102.73, 46.93 LRMS. (ESI) Exact Mass: 259.05, Found: 260.3 (M + H^+).

Compounds 20-26 were synthesized analogously and purified by filtration or preparative TLC, eluting with hexanes/EtOAc. Yields refer to chromatographically and spectroscopically pure compounds.

4-[(3-amino-2-cyano-3-oxoprop-1-en-1-yl)benzamide (20)
Yield: 47 mg (65%). 1H NMR (400 MHz, DMSO) δ 8.22 (s, 1H), 8.12 (br s, 1H), 7.95 – 8.03 (m, 5H), 7.82 (br s, 1H), 7.56 (br s, 1H). 13C NMR (125 MHz, DMSO) δ 167.0, 162.5, 149.7, 137.1, 134.4, 129.8, 128.2, 116.3, 108.2. LRMS (ESI) Exact Mass: 215.07, Found: 216.3 (M + H^+).

2-cyano-3-[(pyridin-4-yl)phenyl]acrylamide (21)
Yield: 33 mg (46%). 1H NMR (400 MHz, DMSO) δ 8.69 (m, 2H), 8.34 (m, 1H), 8.31 (s, 1H), 8.05 - 8.60 (m, 2H), 7.96 (br s, 1H), 7.83 (br s, 1H), 7.70 – 7.76 (m, 3H). 13C NMR (125 MHz, DMSO) δ 162.47, 150.44, 150.36, 145.98, 138.0, 132.88, 130.46, 130.16, 128.59, 121.25, 116.43, 107.65 (one carbon not observed). LRMS (ESI) Exact Mass: 249.09, Found: 250.2 (M + H^+).

3-[(3-amino-2-cyano-3-oxoprop-1-en-1-yl)benzamide (22)
Yield: 36 mg (50%). 1H NMR (400 MHz, DMSO) δ 8.37 (m, 1H), 8.23 (s, 1H), 8.09 (br s, 1H), 8.07 (s, 1H), 8.02 – 8.06 (m, 1H), 7.97 (br s, 1H), 7.81 (br s, 1H), 7.65 (t, 1H, J = 8Hz), 7.54 (br s, 1H). 1H NMR (125 MHz, DMSO) δ 167.1, 162.6, 150.1, 135.3, 132.1, 132.0, 130.8, 129.7, 129.3, 116.2, 107.7. LRMS (ESI) Exact Mass: 215.07, Found: 216.3 (M + H^+).

2-cyano-3-[(isoquinolin-6-yl)acrylamide (23)
Yield: 86 mg (60%). 1H NMR (400 MHz, DMSO) δ 9.42 (s, 1H), 8.63 (d, 1H, J = 5.6 Hz), 8.62 (d, 1H, J = 1.6 Hz), 8.38 (s, 1H), 8.31 (dd, 1H, J = 1.6, 8.4 Hz), 8.14 (d, 1H, J = 8.4 Hz).
solid, filtered and dried, affording 27 mg (92% yield) of cyanoacrylamide suspended in EtOH (3 mL). The reaction mixture was heated at 60 °C for 3 hours. Cyanoacetamide (12.7 mg, 1.5 equiv.) and piperidine•AcOH (2.9 mg, 0.2 equiv.) were suspended in dioxane/water (4:1 v/v, 5 mL). The mixture was degassed and heated to 110 °C for 3 hours. The reaction mixture was then cooled, diluted with EtOAc (50 mL), and filtered through a pad of silica gel with EtOAc washes (20 mL). The filtrate and washes were combined and concentrated. The residue afforded was purified by silica gel chromatography (15:85 → 50:50 → 40:60 Hexanes: EtOAc) to afford 3-(7H-pyrrrolo[2,3-d]pyrimidin-4-yl)benzaldehyde (79 mg, 38% yield, 43) as a white solid. 

1H NMR (400 MHz, CD3OD): 10.14 (s, 1H), 8.84 (s, 1H), 8.62 (s, 1H), 8.41 (d, J = 7.8 Hz, 1H), 8.10 (d, J = 7.7 Hz, 1H), 7.80 (t, J = 7.6 Hz, 1H), 7.59 (d, J = 3.5 Hz, 1H), 6.91 (d, J = 3.5 Hz, 1H); 13C NMR (100 MHz, DMSO-d6): 193.6, 154.1, 152.8, 150.9, 140.1, 138.8, 136.7, 135.5, 134.2, 131.0, 129.9, 128.3, 114.6, 99.6; ESI-MS: 224.14 (MH+).

3-(7H-pyrrrolo[2,3-d]pyrimidin-4-yl)benzaldehyde (22.5 mg, 101 µmol), cyanoacetamide (12.7 mg, 1.5 equiv.) and piperidine•AcOH (2.9 mg, 0.2 equiv.) were suspended in EtOH (3 mL). The reaction mixture was heated at 60 °C for 3 hours and then cooled in an ice bath to precipitate the cyanoacrylamide, which was filtered and dried, affording 27 mg (92% yield) of cyanoacrylamide 27 as a white solid. 1H NMR (400 MHz, DMSO-d6): 8.88 (s, 1H), 8.72 (s, 1H), 8.39 (d, J = 7.8 Hz, 1H),...
8.36 (s, 1H), 8.08 (d, J = 7.7 Hz, 1H), 7.98 (bs, 1H), 7.79 (m, 2H), 7.72 (m, 1H), 7.04 (m, 1H); 13C NMR (100 MHz, DMSO-d6): 162.7, 154.2, 152.7, 150.9, 150.3, 138.8, 132.6, 132.1, 131.4, 129.9, 129.6, 128.2, 116.4, 114.5, 107.5, 99.8; ESI-MS: 290.3 (M+).

Cyanocrylamides 28-42 were synthesized by analogous procedures and isolated, or where necessary, purified by silica gel chromatography.

(E)-3-[3-(3-amino-1H-indazol-4-yl)phenyl]-2-cyanoacrylamide (28)
Yield: 0.8 mg (10% over 2 steps). 1H NMR (400 MHz, DMSO-d6): 11.83 (s, 1H), 8.27 (s, 1H), 8.02 (m, 2H), 7.94 (bs, 1H), 7.79 (bs, 1H), 7.69 (m, 2H), 7.33 (m, 2H), 6.86 (m, 1H), 4.28 (s, 2H); 13C NMR (100 MHz, DMSO-d6): 162.7, 150.5, 147.8, 142.2, 140.0, 134.5, 132.8, 132.1, 130.5, 129.3, 129.0, 126.4, 119.5, 116.6, 110.4, 109.5, 107.1; ESI-MS: 304.2 (M+).

(E)-3-[5-(3-amino-1H-indazol-4-yl)furan-2-yl]-2-cyanoacrylamide (29)
Yield: 26.7 mg (39% over 2 steps). 1H NMR (400 MHz, DMSO-d6): 12.02 (bs, 1H), 8.06 (s, 1H), 7.81 (bs, 1H), 7.69 (bs, 1H), 7.54 (d, J = 3.6 Hz, 1H), 7.42-7.33 (m, 4H), 4.93 (s, 2H); 13C NMR (100 MHz, DMSO-d6): 162.6, 157.1, 148.1, 148.0, 142.5, 135.4, 126.0, 123.6, 122.3, 118.8, 116.7, 113.6, 111.6, 109.0, 100.4; ESI-MS: 294.15 (M+).

(E)-3-[4-(3-amino-1H-indazol-4-yl)thiophen-2-yl]-2-cyanoacrylamide (30)
Yield: 65 mg (70%). 1H NMR (400 MHz, DMSO-d6) δ 8.33 (s, 1H), 8.32 - 8.27 (m, 2H), 8.00 - 7.93 (m, 3H), 7.81 (br s, 1H), 7.73 (dd, 1H, J = 7.8, 7.8 Hz), 7.61 (d, 1H, J = 8.4 Hz), 7.48 (dd, 1H, J = 7.0, 8.4 Hz), 7.30 (d, 1H, J = 7.2 Hz); 13C NMR (125 MHz, DMSO-d6) δ 162.66, 150.67, 140.50, 140.16, 132.66, 132.65, 132.53, 131.85, 129.96, 129.32, 129.25, 126.35, 120.78, 119.75, 116.52, 109.99, 107.26. LRMS (ESI) Exact Mass: 288.10, Found: 289.3 (M + H+).

3-(3-(1H-indazol-4-yl)phenyl)-2-cyanoacrylamide (31)
Yield: 11 mg (74%). 1H NMR (400 MHz, DMSO-d6) δ 8.76 (s, 1H), 8.07 (s, 1H), 7.75 (d, 1H, J = 7.2 Hz), 7.65 (d, 1H, J = 8.3 Hz), 7.59 (d, 1H, J = 3.8 Hz), 7.55 (d, 1H, J = 3.8 Hz), 7.51-7.45 (m, 1H); 13C NMR (125 MHz, DMSO-d6) δ 162.73, 157.38, 148.13, 140.50, 135.19, 133.36, 126.11, 124.70, 120.90, 118.24, 118.03, 116.94, 111.76, 111.72, 100.18; LRMS (ESI) Exact Mass: 278.08, Found: 279.2 (M + H+).

3-(5-(1H-indazol-4-yl)furan-2-yl)-2-cyanoacrylamide (32)
Yield: 29 mg (61%). 1H NMR (400 MHz, DMSO-d6) δ 8.75 (s, 0.45H), 8.69 (s, 0.55H), 8.54 (s, 0.45H), 8.50 (s, 0.55H), 8.39 (s, 0.55H), 8.34-8.30 (m, 2H), 8.22 (s, 0.45H), 8.03-7.93 (m, 3H), 7.81 (br s, 1H), 7.73-7.67 (m, 1H); 13C NMR (125 MHz, DMSO-d6) δ
3-[(4-imidazol-4-yl)pyridin-6-yl]thiophen-2-yl]2-cyanoacrylamide (34)
Yield: 37 mg (82%). $^1$H NMR (400 MHz, DMSO) $\delta$ 8.83-8.73 (m, 1H), 8.55-8.46 (m, 2H), 8.43 (s, 1H), 8.37 (d, 1H, $J = 1.5$ Hz), 8.24 (s, 1H), 7.86 (br s, 1H), 7.75 (br s, 1H);
$^{13}$C NMR (125 MHz, DMSO) $\delta$ 163.15, 162.66, 145.30, 144.27, 143.79, 142.73, 142.47, 142.33, 140.75, 140.09, 139.12, 130.74, 136.74, 136.60, 136.57, 131.31, 129.59, 124.83, 124.59, 118.47, 116.71, 103.62, 102.61; LRMS (ESI) Exact Mass: 295.05, Found: 296.1 (M + H$^+$).

2-cyano-3-(3-methyl-1H-pyrazol-4-yl)phenyl]acrylamide (35)
Yield: 50 mg (88%). $^1$H NMR (400 MHz, DMSO) $\delta$ 8.84 (d, 1H, $J = 2.2$ Hz), 8.54 (d, 1H, $J = 2.2$ Hz), 8.32 (s, 1H), 8.31-8.26 (m, 1H), 8.03-7.96 (m, 2H), 7.95 (br s, 1H), 7.82 (br s, 1H), 7.70 (dd, 1H, $J = 7.8$, 7.8 Hz), 7.55 (s, 3H); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 162.59, 152.07, 150.74, 147.66, 141.65, 138.92, 132.76, 130.66, 129.99, 129.03, 128.06, 127.50, 127.39, 116.55, 114.14, 107.31, 12.23; LRMS (ESI) Exact Mass: 303.11, Found: 304.1 (M + H$^+$).

2-cyano-3-(5-methyl-1H-pyrazol-4-yl)]furan-2-yl]acrylamide (36)
Yield: 8 mg (51%). $^1$H NMR (400 MHz, DMSO) $\delta$ 9.07 (d, 1H, $J = 2.1$ Hz), 8.68 (d, 1H, $J = 2.1$ Hz), 8.01 (s, 1H), 7.46 (d, 1H, $J = 3.7$ Hz), 7.43 (d, 1H, $J = 3.7$ Hz), 2.53 (s, 3H); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 162.72, 156.52, 152.04, 147.50, 145.98, 142.03, 135.04, 126.51, 124.54, 117.84, 117.14, 113.91, 109.09, 100.04, 12.08; LRMS (ESI) Exact Mass: 293.09, Found: 294.4 (M + H$^+$).

3-(2-aminoquinazolin-7-yl)phenyl]2-cyanoacrylamide (37)
Yield: 36 mg (65%). $^1$H NMR (400 MHz, DMSO) $\delta$ 9.15 (s, 1H), 8.37 (s, 1H), 8.31 (s, 1H), 8.03-7.90 (m, 4H), 7.82 (br s, 1H), 7.74-7.69 (m, 2H), 7.57 (dd, 1H, $J = 1.6$, 8.3 Hz), 6.93 (br s, 2H); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 162.61, 162.18, 161.23, 152.21, 150.55, 144.34, 140.24, 132.73, 130.89, 130.04, 129.79, 128.77, 128.54, 122.13, 120.92, 118.87, 116.54, 107.40; LRMS (ESI) Exact Mass: 315.11, Found: 316.1 (M + H$^+$).

3-(5-amino-1H-pyrazol-3-yl)]phenyl]2-cyanoacrylamide (38)
Yield: 0.5 mg (1% over 2 steps). $^1$H NMR (400 MHz, DMSO-d6): 8.18 (m, 1H), 7.91 (m, 1H), 7.83 (d, $J = 8.0$ Hz, 1H), 7.76 (app t, $J = 8.3$ Hz, 1H), 7.66 (m, 3H), 7.55 (m, 2H), 7.30 (m, 3H); ESI-MS: 254.17(MH$^+$). Due to low yield there was not enough compound to characterize $^{13}$C spectrum.

(E)-2-cyano-3-[3-(6-oxo-1,6-dihydropyridin-2-yl)phenyl]acrylamide (39)
Yield: 14 mg (35%). $^1$H NMR (400 MHz, DMSO-d6): 8.32 (bs, 1H), 8.26 (s, 1H), 8.03-7.96 (m, 4H), 7.82 (bs, 1H), 7.68 (m, 2H), 7.61 (m, 1H), 6.47 (m, 1H); $^{13}$C NMR (100 MHz, DMSO-d6): 163.3, 162.4, 158.6, 150.2, 147.9, 140.8, 135.8, 132.4, 130.3, 130.1, 129.6, 128.7, 116.3, 107.5, 106.7; ESI-MS: 266.18 (MH$^+$).
3-(3-(1,6-naphthyridin-8-yl)phenyl)-2-cyanoacrylamide (40)
Yield: 69 mg (92%). $^1$H NMR (400 MHz, DMSO) $\delta$ 9.47 (s, 1H), 9.17 (dd, 1H, $J = 1.8, 4.2$ Hz), 8.84 (s, 1H), 8.70 (dd, 1H, $J = 1.8, 8.2$ Hz), 8.34-8.31 (m, 1H), 8.29 (s, 1H), 8.06-7.93 (m, 3H), 7.82-7.77 (m, 2H), 7.76-7.70 (m, 1H); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 163.04, 155.52, 153.65, 150.78, 147.28, 146.12, 136.77, 136.57, 134.79, 132.26, 132.06, 131.98, 129.56, 129.26, 123.52, 123.49, 116.61, 107.19; LRMS (ESI) Exact Mass: 300.10, Found: 301.34 (M + H$^+$).

3-(5-(1,6-naphthyridin-8-yl)furan-2-yl)-2-cyanoacrylamide (41)
Yield: 50 mg (quant.). $^1$H NMR (400 MHz, DMSO) $\delta$ 9.45 (s, 1H), 9.38 (s, 1H), 9.30 (dd, 1H, $J = 1.7, 4.3$ Hz), 8.71 (dd, 1H, $J = 1.7, 8.2$ Hz), 8.13 (d, 1H, $J = 3.7$ Hz), 8.09 (s, 1H), 7.86 (dd, 1H, $J = 4.3, 8.2$ Hz), 7.53 (d, 1H, $J = 3.7$ Hz); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 162.56, 155.55, 153.96, 153.02, 147.70, 145.41, 143.78, 137.02, 135.16, 124.21, 123.68, 123.09, 120.17, 117.62, 116.96, 101.47; LRMS (ESI) Exact Mass: 290.08, Found: 291.1 (M + H$^+$).

(E)-2-cyano-3-[1-(6-methylpyrazin-2-yl)piperidin-4-yl]acrylamide (42)
Yield: 8.7 mg (7%). $^1$H NMR (400 MHz, CD$_3$OD): 7.98 (s, 1H), 7.64 (s, 1H), 7.34 (d, $J = 10.0$Hz, 1H), 4.58 (bs, 2H), 4.46 (m, 2H), 3.07-2.85 (m, 3H), 2.36 (s, 3H), 1.85 (m, 2H), 1.60 (m, 2H); $^{13}$C NMR (125 MHz, DMSO-d6): 162.2, 160.2, 153.8, 150.1, 131.4, 128.2, 114.9, 111.4, 43.0, 29.2, 21.3; ESI-MS: 272.22 (MH$^+$).
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