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Discovery of 2-(Cyclohexylmethylamino)pyrimidines as a New Class of Reversible Valosine Containing Protein Inhibitors

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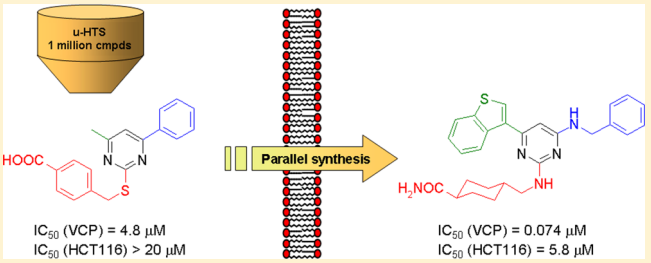
Supporting Information

ABSTRACT: Valosine-containing protein (VCP), also known as p97 or cdc48 in yeast, is a highly abundant protein belonging to the AAA ATPase family involved in a number of essential cellular functions, including ubiquitin−proteasome mediated protein degradation, Golgi reassembly, transcription activation, and cell cycle control. Altered expression of VCP has been detected in many cancer types sometimes associated with poor prognosis. Furthermore, VCP mutations are causative of some neurodegenerative disorders. In this paper we report the discovery, synthesis, and structure−activity relationships of substituted 2-aminopyrimidines, representing a new class of reversible VCP inhibitors. This class of compounds, identified in a HTS campaign against recombinant VCP, has been progressively expanded and manipulated to increase biochemical potency and gain cellular activity.



INTRODUCTION

Valosine-containing protein (VCP/p97), known as cdc48 in yeast, is a member of the AAA (ATPases associated with various cellular activities) family of ATPases[1] and is an essential and highly abundant protein in cells. VCP may be considered an enzymatic machine that transfers, through adaptor proteins, the energy derived from ATP hydrolysis to the substrates to perform a wide range of cellular functions such as endoplasmic reticulum associated degradation (ERAD), Golgi reassembly, transcription regulation, and cell cycle control.[2] VCP is characterized by a highly flexible hexameric structure with subunits arranged as a ring around a central pore. Each subunit is composed of an N-terminal domain responsible for the binding to adaptor proteins and two AAA domains called D1 and D2 that sustain nucleotide binding and hydrolysis.[3,4] The structure and the role of the C-terminal extension still remain elusive.

The pivotal role of this protein in many essential cellular functions suggests that altered expression or mutation of VCP may lead to pathological consequences. Consistent with this finding, several reports indicate that elevated levels of VCP have been detected in many cancer types sometimes associated with poor prognosis.[5,6] Moreover, several data demonstrate an involvement of VCP in neurodegenerative diseases.[7] The increasing interest around this new potential molecular target has prompted different groups to undertake screening programs in the attempt to identify small molecules able to inhibit VCP enzymatic activity. This would facilitate a better understanding of the effective relevance of this target in cancer therapeutics. A few molecules interfering with VCP functions were previously reported in literature.[8−11] Recently, a class of potent and specific allosteric inhibitors have been described by our group.[12] Here we report the synthesis and the structure−activity relationships of substituted 2-aminopyrimidines, a new class of reversible VCP inhibitors, acting through a different mechanism of action. The original hit **1**, discovered after a HTS campaign against recombinant VCP, was the 2-alkylsulfanylpyrimidine shown in Figure 1.

This compound inhibited VCP ATPase activity with an IC_{50} of 4.8 μM in standard assay conditions. Serial dilution experiments indicated a reversible mode of binding and mass

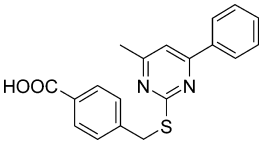


Figure 1. Structure of 2-alkylsulfanylpyrimidine **1**.

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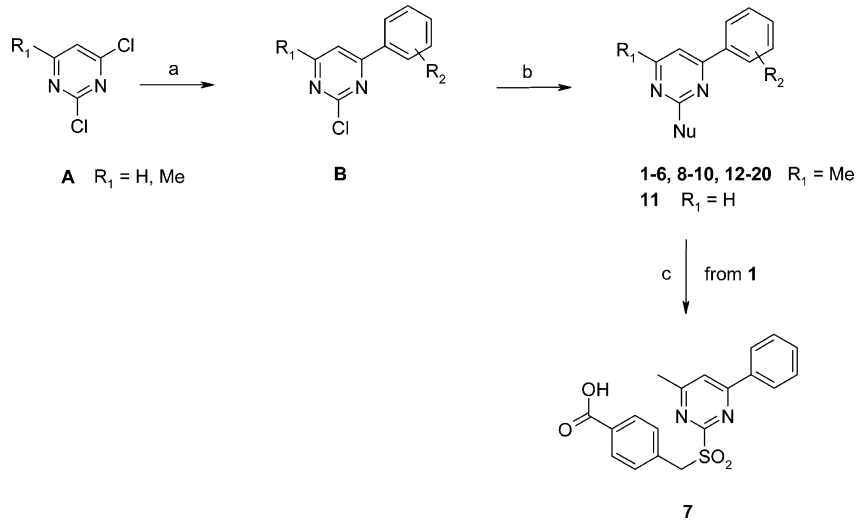
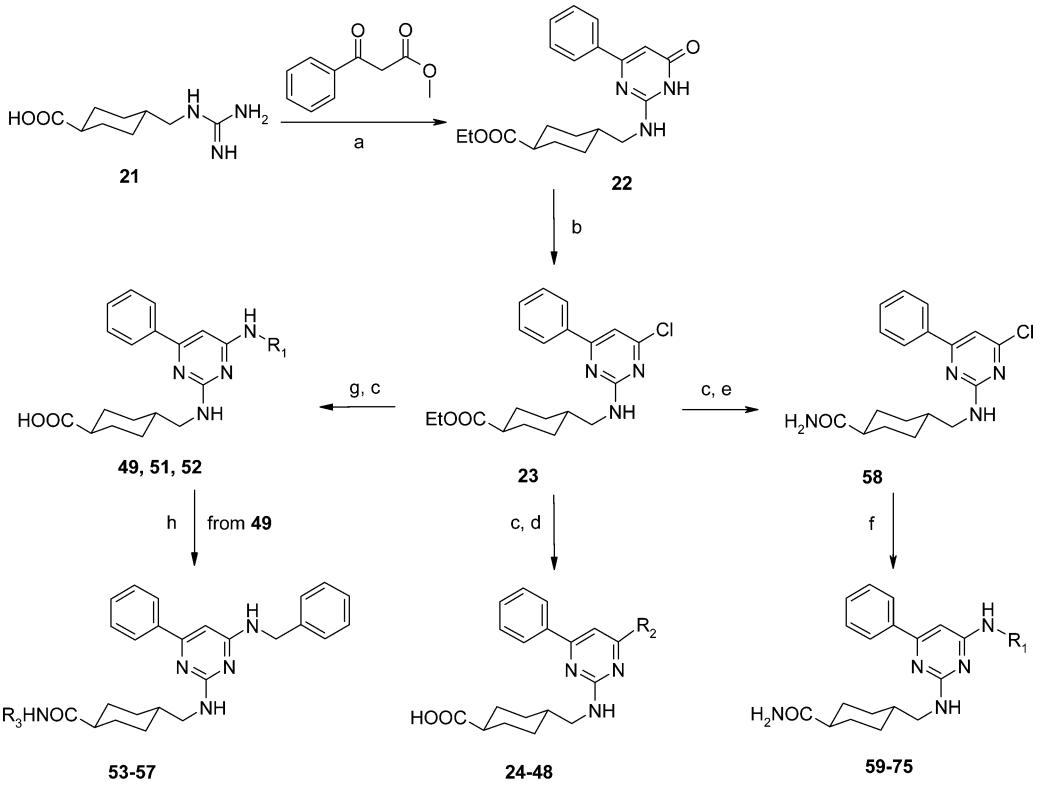
Scheme 1[a]



[a]Reagent and conditions: (a) $ArB(OH)_2$, $PdCl_2(dppf)$, Cs_2CO_3, dioxane/H_2O, 70 °C, 15 min; (b) nucleophile (Nu), DBU, dioxane, 130−150 °C, 2−3 h; (c) *m*-chloroperoxybenzoic acid, THF, rt, 3 h.

Scheme 2[a]



[a]Reagent and conditions: (a) 180 °C, 2 h, then EtOH, H_2SO_4 cat., 78 °C, 1 h; (b) $POCl_3$, *N*,*N*-dimethylaniline, 110 °C, 1 h; (c) LiOH, THF/H_2O/MeOH 1:1:1, rt, 15 h; (d) $R_2B(OH)_2$, $PdCl_2(dppf)$, Cs_2CO_3, dioxane/H_2O, 100 °C, 1 h; (e) NH_4OBt, EDC, DIPEA, DCM/DMF 10:1, rt, 15 h; (f) R_1NH_2, DIPEA, NMP, 220 °C, 1 h; (g) R_1NH_2, DIPEA, ACN, 180 °C, 1 h; (h) HOBt, EDC, R_3NH_2, DIPEA, DCM, rt, 6 h.

spectrometry analysis of VCP upon incubation with molar excess of the inhibitor did not detect any modification of the protein (data not shown). To better understand the mechanism of action of this inhibitor, the IC_{50} of compound **1** was also evaluated at a saturating ATP concentration (1 mM). In these conditions, a 10-fold increase in the IC_{50} value was observed, indicating that this compound is indeed sensitive to ATP concentration (Supporting Information, Figure S1, p S3). This might suggest a direct competition for the active site. Alternatively, given the complex and cooperative mechanism of VCP enzymatic activity, one cannot exclude that the binding of the compound outside of the ATP pocket may affect the binding affinity for ATP. Starting from compound **1**, a medicinal chemistry program was set up to identify the key structural features responsible for activity and to improve the biological profile of this class of compounds.

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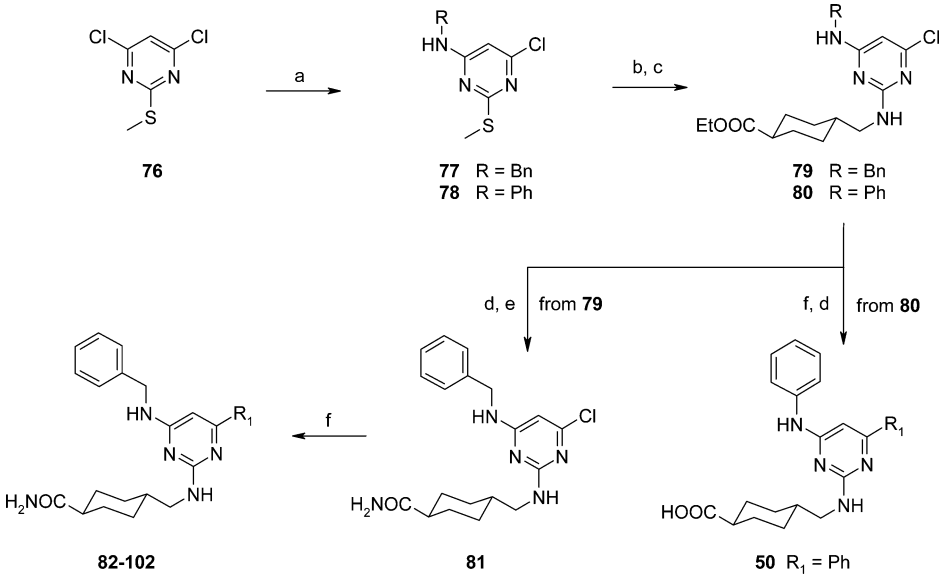
Scheme 3[a]



[a]Reagent and conditions: (a) benzylamine, DIPEA, EtOH, 120 °C, 2 h; (b) *m*-chloroperoxybenzoic acid, DCM, rt, 3 h; (c) *trans*-4-aminomethylcyclohexanecarboxylic acid ethyl ester, NMP, 180 °C, 15 min; (d) LiOH, THF/H_2O/MeOH 1:1:1, rt, 15 h; (e) NH_4OBt, EDC, DIPEA, DCM/DMF 10:1, rt, 15 h; (f) $R_1B(OH)_2$, $PdCl_2(dppf)$, Cs_2CO_3, dioxane/H_2O, 100 °C, 1 h.

CHEMISTRY

Two main approaches were used to synthesize the compounds described in this paper: (1) progressive substitution of commercially available pyrimidines such as 2,4-dichloro- and 2,4-dichloro-6-methylpyrimidine (Scheme 1) or 4,6-dichloro-2-methylsulfanylpyrimidine (Scheme 3); (2) formation of the pyrimidine ring by condensation of acetoacetates with previously functionalized guanidines, as depicted in Scheme 2 for methyl benzoylacetate and 4-guanidinomethylcyclohexanecarboxylic acid **21**.[13,14]

Compounds **1**–**20** were prepared by a Suzuki coupling between 2,4-dichloropyrimidine derivatives **A** and the appropriate boronic acid/ester, providing the corresponding 2-chloro-4-phenylpyrimidines **B** with good site selectivity (Scheme 1).[15]

Intermediates **B** were then treated under microwave heating with different thiol and amine nucleophiles to give the desired compounds. Sulfone **7** was obtained from compound **1** after oxidation with *m*-chloroperoxybenzoic acid.

Ester **23** (Scheme 2) was found to be a very versatile intermediate for expansion of the 4-[(4-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid scaffold.

The pyrimidine core was synthesized by cyclization of 4-guanidinomethylcyclohexanecarboxylic acid **21** with methyl benzoylacetate. Subsequent esterification provided compound **22** that upon treatment with $POCl_3$ afforded the 4-chloro derivative **23**. Suzuki reaction on **23** and subsequent hydrolysis gave compounds **24**–**48**. Compounds **49**, **51**, and **52** were obtained from the same intermediate by microwave irradiation in the presence of the corresponding amines and hydrolysis of the ester group. The carboxylic acid **49** was in turn condensed with the appropriate nucleophile to prepare compounds **53**–**57**. Intermediate **23** was also used for multigram scale preparation of compound **58** that was obtained via hydrolysis and condensation with EDC/NH_4OBt. From **58** and the suitable benzylamine, compounds **59**–**75** were then prepared by microwave irradiation.

An alternative approach (Scheme 3) was exploited for preparation of compounds **82**–**102**.

Reaction of 4,6-dichloro-2-methylsulfanylpyrimidine **76** with both benzylamine and aniline, under controlled conditions, afforded the monosubstituted intermediates **77** and **78**, respectively, in good yield (>90%). Oxidation with *m*-chloroperoxybenzoic acid and displacement of the resulting methansulfonyl derivatives with *trans*-4-aminomethylcyclohexanecarboxylic acid ethyl ester gave the chloro derivatives **79** and **80**. Intermediate **80** was reacted under Suzuki conditions with phenylboronic acid and then hydrolyzed to provide compound **50**. Intermediate **79** was transformed in the corresponding primary amide **81** that was reacted with a series of boronic acid derivatives affording compounds **82**–**102**.

RESULTS AND DISCUSSION

A first set of derivatives of compound **1** (Table 1) was designed with the aim of understanding the key features responsible for the biochemical activity of this class of compounds.

Considering the sensitivity of **1** to ATP concentration, compound **1** was overlapped with the ADP molecule in its VCP/D2 binding conformation (Figure 2a,b) extracted from a published crystallographic complex (PDB code 3CF0).[4] The resulting superimposition was performed in order to maximize the conformational fitting of the two molecules at the adenine region, with the carboxylate group of compound **1** overlapped with the β-phosphate group of ADP. The asymmetric substitution of the pyrimidine ring resulted in two possible conformation alignments, differing by 180° rotation around the pyrimidine–sulfur bond.

Ab initio geometry optimization of compound **1** (see Experimental Section) resulted in a minimum showing an "out of plane" of ±20° for the phenyl attached to the pyrimidine ring. As previously reported for another class of VCP inhibitors,[12] cocrystallization trials proved to be very problematic because of the high molecular weight and flexibility of the protein. For this reason, structure-based design

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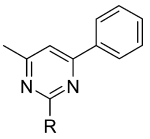
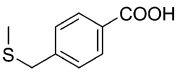
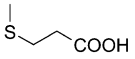
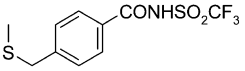
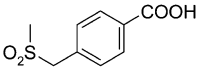
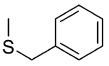
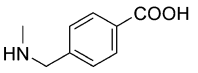
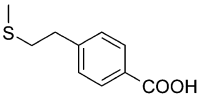
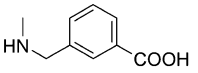
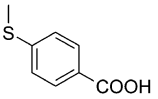
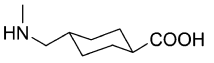
Table 1. SAR: Variations at the Acidic Moiety



ID	R	VCP IC_{50}(uM)	ID	R	VCP IC_{50}(uM)
1		4.8	**6**		> 37
2		21.6	**7**		> 37
3		> 37	**8**		15.4
4		20.5	**9**		> 37
5		> 37	**10**		2.6

optimization of compound **1** was not feasible and instead we focused on a ligand-based approach for the medicinal chemistry expansion.

The carboxylic acid group appeared to be essential. Its removal (compound **3**) caused a drop in activity. On the other hand, compound **2**, which still retains one acidic hydrogen, maintained marginal activity. Considering the proposed compound **1**/ADP ligand alignment, the length and the nature of the spacer between the central scaffold and the carboxylic group also appeared to be an important structural feature. Compounds **4** and **5**, whose spacers are respectively one carbon atom longer and shorter than the original compound, were significantly less active. The same also applies to compound **6**, which lacks the central phenyl ring. This indicates that the binding of compound **1** to the active site is not purely due to a nonspecific electrostatic interaction of the carboxylic acid group with the protein. Another important aspect that was considered during the early stage expansion was the isosteric replacement of the sulfur atom at position 2 of the pyrimidine ring. This was done both to simplify the synthetic procedure, widening the array of potential substituents, and to prevent the possible metabolic oxidation of sulfur, which would lead to the corresponding inactive sulfone **7**. Analog **8**, with nitrogen in place of sulfur, was 3-fold less active than the parent compound but still retained residual activity, unlike the inactive meta-isomer **9**. This, once again, indicates that the carboxylic group must be correctly positioned. On the other hand, the reduced biochemical activity of **9** was widely recovered by replacing the phenyl ring of the spacer with a cyclohexyl ring, as in derivative **10**. In this case, the carboxylic group in trans-relationship with the amino group is likely to better interact with the protein. Thus, the 2-aminopyrimidine **10** represented a profitable framework for further chemical expansion and optimization. In this respect, a limited number of derivatives was first synthesized in order to map the phenyl ring at position 6 (Table 2).

As evident from the table, ortho-substitution, likely affecting the planarity of the system, seemed to be particularly detrimental for activity (**12**, **13**). As for the remaining compounds, none of the substituents considered led to significant improvements. On the other hand, the methyl at position 4 seemed to hold some importance. Its removal caused a moderate decrease of activity (**11**), while its replacement with an additional phenyl ring boosted activity to the submicromolar range. In fact, compound **24** was 10-fold more active than the parent methyl analog **10**. Superimposition of compound **24** to the reference ADP structure showed a good conformational fit (Figure 2c). The replacement of the phenyl with a cyclohexyl ring allowed a more flexible placement of the terminal carboxylate moiety maintaining an extended molecular conformation.

The subsequent expansion was therefore planned on compound **24**, decorating only one of the two phenyl rings (Table 3). Also in this series, substitutions on the phenyl ring generally turned out to be detrimental with the exception of a few compounds including **33** and **34**, characterized by having H-bond acceptor groups such as methylsulfonyl and nitro at the meta-position, or compound **42** with an amino group at the para-position. Interestingly, the combination of the two arrangements, as in derivative **43**, led to a further increase in potency with an IC_{50} of 60 nM. The replacement of one of the two phenyls with a heterocyclic moiety (**44**−**48**) did not prove to be effective except for the indolyl derivative **44** that led to modest improvement of activity. The insertion of nitrogen between the central pyrimidine scaffold and one of the phenyls was very advantageous, as in the benzylamino (**49**) and phenylamino (**50**) derivatives that improved potency by 1 order of magnitude as compared to the parent compound **24**. By contrast, the alkylamino derivatives **51** and **52** were disappointing.

To exclude a potential interference of this class of compounds on the global structure of the hexamer, native gel analysis of VCP in the presence of derivative **43** and the parent

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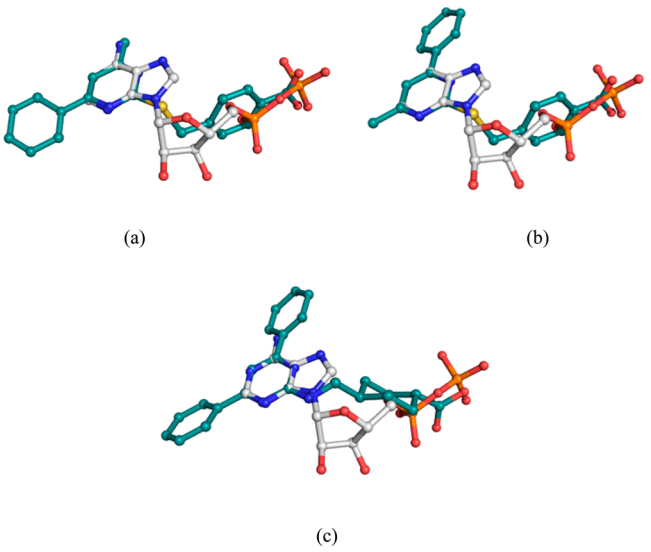
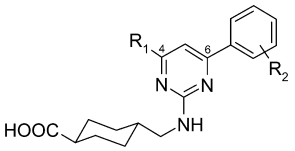


Figure 2. (a, b) Compound **1** superimposed onto ADP. Two alignments are possible because of asymmetric substitution of the pyrimidine ring. (c) Compound **24** superimposed onto ADP. Compounds **1** and **24** are represented with cyan C atoms, and the phenyl/pyrimidine reciprocal orientation is set at 20°. ADP crystallographic conformation, extracted from the 3CF0 complex, is reported with gray C atoms.

Table 2. SAR: Methyl Series



compd	R_1	R_2	VCP IC_{50} (μM)
10	Me	H	2.6
11	H	H	8.7
12	Me	*o*-Me	>37
13	Me	*o*-OMe	>37
14	Me	*m*-Me	11.4
15	Me	*m*-Cl	4.8
16	Me	*m*-OMe	>37
17	Me	*p*-Me	2.7
18	Me	*p*-Cl	2.6
19	Me	*p*-F	8.4
20	Me	*p*-OMe	>37
24	Ph	H	0.3

compound **1** was performed, showing that these inhibitors do not affect the oligomeric state of VCP (Supporting Information, Figure S2, p S4). Furthermore, limited proteolysis experiments indicated that the two compounds display a potency-related protective effect on VCP from trypsin digestion, as reported for allosteric VCP inhibitors previously described (Supporting Information, Figure S3, p S5).[16] Compound **1** and **43** were also profiled against other representative AAA ATPases (NSF, SPATA5, VPS4B, and RuvBL1), HSP90, and a panel of 50 kinases[16] and did not display cross-reactivity up to the highest tested concentration of 10 μM (data not shown).

To identify the binding site of this class of inhibitors, we synthesized the azido derivative **35** (Table 3) as a probe for photoaffinity experiments. After incubation with wild-type VCP protein and UV irradiation, subsequent LC/MS analysis revealed that VCP was derivatized by a single molecule of azido probe (Supporting Information, Figure S4b, p S6). VCP digestion with Asp-N protease and ESI-MS/MS of the originated peptides (Supporting Information, Figure S4c, p S7) allowed the identification of the derivatized amino acid as Asp478. The specificity of the labeling azido probe was confirmed by competition experiments with the close analog **43** that completely prevented VCP protein derivatization. Asp478 is located on the D1−D2 linker of VCP in proximity to the D2 ATP-binding site (Figure 3a).

With regard to compound **49**, we assumed that the observed improvement of potency might be explained in terms of possible engagement of additional stabilizing binding interactions with the protein. In order to investigate the possible binding mode and interactions in comparison to ADP, compound **49** was docked in the VCP/D2 binding site (Experimental Section). The obtained docking pose (Figure 3b) shows that compound **49** is slightly displaced relative to the ADP binding conformation, probably to better accommodate the two bulky phenyl rings. The benzyl group is projected in a small hydrophobic tunnel behind the amino group of the adenine, while the phenyl ring is at the entrance of the cavity in proximity to Asp478.

In this pose, alternative intermolecular hydrogen bonds are observed between the 6-aminopyrimidine moiety and Asn660/Thr688 side chains, while the terminal carboxylate group reproduces the ADP β-phosphate interactions (hydrogen bonds and Coulomb charge/charge) with residues Gly521, Cys522, Gly523, Lys524, and Thr525 (Figure 3b). Despite the different interactions pattern, compound **49** fits the VCP/D2 binding cavity and is able to occupy almost the same space as ADP.

As expected, compounds **49** and **50**, despite having nanomolar biochemical activity, did not show a significant antiproliferative effect (IC_{50} > 20 μM on HCT-116 cell line), likely because of the presence of the carboxylic acid group that generally hampers cell permeability. As a result, subsequent efforts were directed toward the identification of carboxylic acid replacements able to maintain acceptable biochemical activity and induce permeability. This study was carried out on the aminobenzylic series, and a limited number of compounds were synthesized (**53**−**57**, Table 4), all of them possessing at least one acidic proton.

All compounds retained biochemical activity below 1 μM, even though with a marked reduction when compared to the parent carboxylic acid **49**. It is worth mentioning that the corresponding methyl ester, tested in the same conditions, was inactive (data not shown). For some of these derivatives however we were able to obtain, for the first time within this class, borderline cellular activity on the HCT-116 cell line, as in the case of the two carboxamido derivatives **53** and **54**. The primary carboxamide **53**, endowed with better potency, was finally chosen for further chemical expansion. First, we wanted to investigate the effect of substitutions on the two aromatic rings branching off the central pyrimidine scaffold. The first one explored was the aminobenzylic ring that was extensively decorated with canonical substituents (Table 5). As observed for other series, the substitution of the aminobenzylic ring, independent of the substituent considered, led to substantial reduction of biochemical activity (**59**−**75**). The docking pose of compound **49** helped rationalize these results: the aminobenzyl moiety is projected in a small rigid hydrophobic tunnel with limited space available (Figure 3a). Almost all the explored substitutions decreased biochemical potency likely

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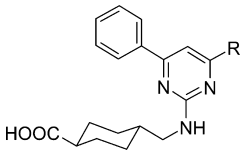
Table 3. SAR: Phenyl Series



compd	R	VCP IC_{50} (μM)	compd	R	VCP IC_{50} (μM)
24	Ph	0.261	**39**	Ph-*p*-OMe	0.921
25	Ph-*o*-Me	0.673	**40**	Ph-*p*-SO_2Me	1.091
26	Ph-*o*-F	0.141	**41**	Ph-*p*-NO_2	2.189
27	Ph-*o*-Cl	0.700	**42**	Ph-*p*-NH_2	0.112
28	Ph-*o*-OMe	3.041	**43**	Ph-3-NO_2-4-NH_2	0.060
29	Ph-*m*-Me	0.578	**44**	5-indolyl	0.150
30	Ph-*m*-F	0.552	**45**	2-furanyl	0.570
31	Ph-*m*-Cl	0.483	**46**	3-pyridyl	1.201
32	Ph-*m*-OMe	0.543	**47**	4-pyridyl	1.667
33	Ph-*m*-SO_2Me	0.116	**48**	3-furanyl	2.188
34	Ph-*m*-NO_2	0.201	**49**	NHBn	0.027
35	Ph-*m*-N_3	0.879	**50**	NHPh	0.036
36	Ph-*p*-Me	0.577	**51**	NH-cyclohexyl	4.313
37	Ph-*p*-F	0.742	**52**	N-morpholinyl	>10
38	Ph-*p*-Cl	0.221			

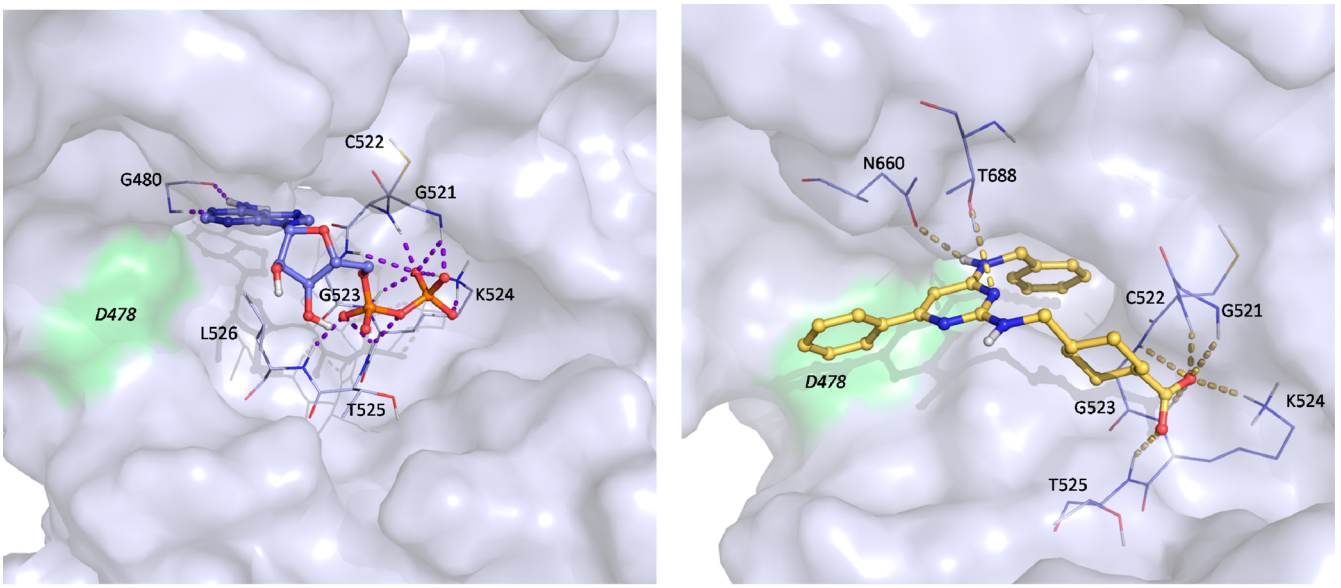


Figure 3. (a) Position of Asp478 (highlighted in green) relative to ADP in the VCP/D2 binding site (3CF0 X-ray structure). (b) Intermolecular hydrogen bonds monitored for compound **49** docked in the VCP/D2 binding site.

because they disturb the placement of the phenyl ring or because of the steric clash with the pocket. Only meta-substitution with a small atom such as fluorine seemed to maintain activity comparable with compound **53**. In this case, fluorine, acting as a H-bond acceptor, could foster an interaction with Gly480.

A better outcome was obtained introducing variations on the other side of the molecule (Table 6).

In this case substitutions on the phenyl ring (**82**–**95**), although not leading to significant improvements, maintained biochemical activity in the same range as the reference compound **53**. Some interesting results were obtained by replacing the phenyl with heterocyclic rings (**96**–**102**). The best of these examples had biochemical IC_{50} below 100 nM and antiproliferative activity on HCT-116 cell line in the low micromolar range (**96**–**99**). Once again, the docking pose of compound **49** helped rationalize these results; the phenyl ring is indeed projected outside the cavity, where the available space is more extended and where many flexible polar/charged side chains could establish additional interactions with substituents on the phenyl ring.

In order to associate antiproliferative activity to VCP inhibition, modulation of the most sensitive VCP biomarkers was assessed upon treatment of HCT-116 cells with one of the most active compounds (**98**). Previous siRNA experiments[16] had in fact shown that VCP ablation induced accumulation of polyubiquitinated proteins, as well as activation of the unfolded protein response as indicated by induction of C/EBP

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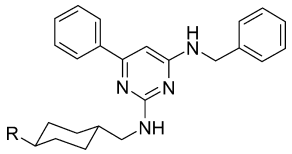
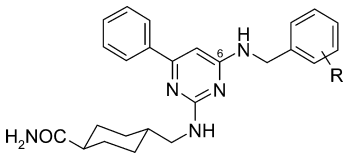
Table 4. SAR: Replacement of the Carboxylate Group



compd	R	VCP IC_{50} (μM)	HCT116 IC_{50} (μM)
49	COOH	0.027	>20
53	$CONH_2$	0.127	9.58
54	CONHMe	0.448	9.26
55	$CONHSO_2Me$	0.133	>20
56	$CONHSO_2CF_3$	0.264	>20
57	$CONHNH_2$	0.986	>20

Table 5. SAR: Variations at the Benzylamino Moiety at Position 6



compd	R	VCP IC_{50} (μM)	compd	R	VCP IC_{50} (μM)
53	H	0.127	**67**	*m*-OMe	5.645
59	*o*-Me	9.288	**68**	*m*-NO_2	4.608
60	*o*-F	1.691	**69**	*m*-SO_2NH_2	7.610
61	*o*-Cl	6.939	**70**	*p*-Me	2.181
62	*o*-Br	7.108	**71**	*p*-F	2.433
63	*o*-OMe	8.860	**72**	*p*-Cl	3.473
64	*m*-Me	1.679	**73**	*p*-OMe	5.036
65	*m*-F	0.474	**74**	*p*-SO_2NH_2	5.165
66	*m*-Cl	2.762	**75**	*p*-SO_2Me	>10

homologous protein transcription factor (CHOP, also known as Gadd153). A similar outcome was observed with another class of allosteric VCP inhibitors recently disclosed.[12] Interestingly, immunoblot analysis of HCT-116 cells treated with the representative compound **98** indicates a dose-dependent accumulation of polyubiquitinated proteins and induction of CHOP, validating the potential for this class of compounds to reach potency and permeability features necessary to inhibit VCP also in cellular contexts (Figure 4).

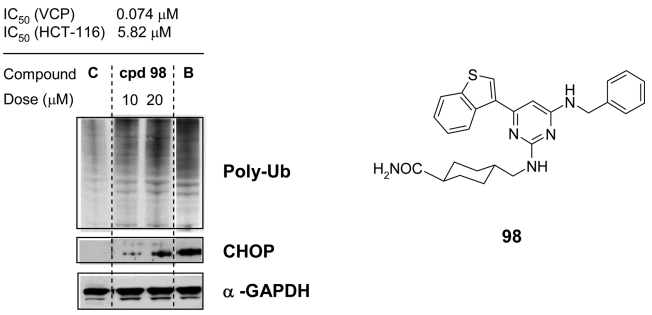


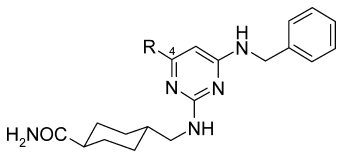
Figure 4. Analysis of biomarkers modulation upon treatment with VCP inhibitor **98**.

HCT-116 cells were treated with increasing doses of inhibitor for 8 h. Cells were then rinsed with PBS and lysed. An amount of 20 μg of cell lysate proteins was loaded, and proteins were fractionated in SDS–PAGE and subjected to immunoblotting with the indicated antibodies. C stands for control. B stands for the proteasome inhibitor bortezomib used as a standard.

Compound **98** was also profiled against a panel of 50 kinases and did not display any measurable cross-reactivity up to 10 μM concentration (data not shown).

Analysis of ligand efficiency (LE) metrics[17] has been performed for the compounds synthethized after this preliminary stage of expansion. Data (Supporting Information, Table S1, p S9) indicate that LE of the original hit was generally maintained for the most representative compounds after each round of optimization, while LLE was improved. Additional work clearly needs to be spent in lead optimization stage to improve the biological profile and the physicochemical properties of this chemical class. Nevertheless this result underlines the possibility of inhibiting VCP cellular functions by means of compounds endowed with different mechanisms of action, thus widening the spectrum of druggability of this target.

Table 6. SAR: Variation at the Aromatic Group at Position 4



compd	R	VCP IC_{50}(μM)	HCT116 IC_{50} (μM)	compd	R	VCP IC_{50} (μM)	HCT116 IC_{50} (μM)
53	Ph	0.127	9.58	**92**	Ph-*p*-Cl	0.106	7.32
82	Ph-*o*-F	0.046	>10	**93**	Ph-*p*-CF_3	0.222	7.61
83	Ph-*m*-$COCH_3$	0.087	6.55	**94**	Ph-*p*-OEt	0.246	2.23
84	Ph-*m*-OH	0.106	>10	**95**	Ph-*p*-F	0.299	>10
85	Ph-*m*-CH_2OH	0.135	>10	**96**	2-benzofuranyl	0.061	4.64
86	Ph-*m*-OMe	0.149	6.66	**97**	5-indazolyl	0.071	5.72
87	Ph-*m*-Me	0.183	4.16	**98**	3-benzothiophenyl	0.074	5.82
88	Ph-*m*-CF_3	0.237	7.97	**99**	5-indolyl	0.082	5.64
89	Ph-*p*-$NHCOCH_3$	0.059	4.31	**100**	3-pyridyl	0.109	>10
90	Ph-*p*-CH_2OH	0.089	7.89	**101**	3-thiophenyl	0.452	7.41
91	Ph-*p*-$COCH_3$	0.102	>10	**102**	3-furanyl	0.491	8.75

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CONCLUSIONS

2-Alkylsulfanylpyrimidine **1**, which emerged after a HTS campaign against recombinant VCP as a low micromolar activity hit devoid of antiproliferative effect, was extensively manipulated to improve potency and acquire cellular activity. The initial replacement of sulfur atom at position 2 with nitrogen and the progressive optimization of the scaffold at three different positions led to a series of low nanomolar inhibitors of VCP ATPase activity. Replacement of the carboxylic acid group with cell permeable surrogates, in particular a primary carboxamide, led to establishment of cellular activity while maintaining potency. Further chemical expansion led to the identification of a small series of derivatives endowed with low micromolar antiproliferative activity. Moreover, for representative compound **98** we could demonstrate modulation of main biomarkers associated with VCP ablation. Additional work will be undertaken to elucidate more in depth the mode of action of this class of compounds to further improve biochemical potency and cellular activity and to understand the real potential of this class in anticancer therapeutics through the optimization of the physicochemical profile.

EXPERIMENTAL SECTION

1. Chemistry. Unless otherwise noted, solvents and reagents were obtained from commercial suppliers and used without further purification. All reactions involving air- or moisture-sensitive reagents were performed under an argon atmosphere. All final compounds were purified to >95% purity as determined by high-performance liquid chromatography (HPLC). Purity was measured by HPLC on a Waters X Terra RP18 (4.6 mm × 50 mm, 3.5 μm) column using a Waters 2790 HPLC system equipped with a 996 Waters PDA detector and Micromass model A ZQ single quadrupole mass spectrometer, equipped with an electrospray ion source (ESI). Mobile phase A was an ammonium acetate 5 mM buffer (pH 5.5 with acetic acid/acetonitrile 95:5), and mobile phase B was H_2O/acetonitrile (5:95). The following conditions were used: a gradient from 10% to 90% B in 8 min and held at 90% B for 2 min; UV detection at 220 and 254 nm; a flow rate of 1 mL/min; an injection volume of 10 μL; full scan, mass range from 100 to 800 amu. The capillary voltage was 2.5 kV. The source temperature was 120 °C. The cone was 10 V. Masses are given as an m/z ratio. Column chromatography was conducted either under medium pressure on silica gel (Merck silica gel 40−63 μm) or on prepacked silica gel cartridges (Biotage) on a Horizon system. When necessary, compounds were purified by preparative HPLC on a Waters X Terra Prep RP18 (19 mm × 100 mm, 5 μm) column using a Waters FractionLynx system equipped with a Waters 2996 PDA detector and a Waters ZQ single quadrupole mass spectrometer, with electrospray ionization, in the positive mode. Mobile phase A was water and 0.05% NH_4OH (pH 10)/acetonitrile 95/5, and mobile phase B was acetonitrile. The following conditions were used: a gradient from 10% to 90% B in 8 min and held at 100% B for 2 min; a flow rate of 20 mL/min. ^{1}H NMR spectra were acquired at 25 °C in DMSO-d_6 on a Varian Inova 500 spectrometer operating at 499.7 MHz and equipped with a 5 mm ^{1}H{^{13}C,^{15}N} z-axis-PFG indirect detection probe and at 28 °C on a Varian Inova 400 spectrometer operating at 400.5 MHz and equipped with a 5 mm ^{1}H{^{15}N−^{31}P} z-axis-PFG indirect detection probe. Residual not-deuterated DMSO-d_6 signal was used as reference (δ = 2.50 ppm for ^{1}H and δ = 39.5 ppm for ^{13}C). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, bs = broad singlet, dd = doublet of doublet, td = triplet of doublet, m = multiplet), coupling constants, and number of protons. Standard two-dimensional sequences provided by Varian (gradient-enhanced HMBC ^{1}H−^{13}C and T-ROESY) were used for structure determinations (Supporting Information, p S33, S36, S38). Low-resolution mass spectrometry (MS) data were determined on a Finnigan MAT LCQ ion trap instrument, equipped with ESI. ESI high-resolution mass spectrometry (HRMS) results were obtained on a Waters Q-Tof Ultima directly connected with micro HPLC 1100 Agilent as previously described. Thin-layer chromatography was performed on Merck silica gel 60 plates coated with 0.25 mm layer with fluorescent indicator. Components were visualized by UV light (λ = 254 and 366 nm) and iodine vapors. All reactions requiring microwave heating were performed in Biotage Initiator Sixty.

The following abbreviations for solvents and reagents are used: Pd(dppf)Cl_2 = [1,1′-bis(diphenylphosphino)ferrocene]-dichloropalladium(II), DBU = 1,5-diazabiciclo[5.4.0]undec-5-ene, DCM = dichloromethane, THF = tetrahydrofuran, MeOH = methanol, EtOH = ethanol, DIPEA = *N*-ethyldiisopropylamine, ACN = acetonitrile, EDC = 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, NH_4OBt = 1-hydroxy-1*H*-benzotriazole ammonium salt, DMF = dimethylformamide, NMP = *N*-methyl-2-pyrrolidone, AcOEt = ethyl acetate.

4-(4-Methyl-6-phenylpyrimidin-2-ylsulfanylmethyl)benzoic Acid (1). In a microwave vial 2,4-dichloro-6-methylpyrimidine (200 mg, 1.23 mmol), phenylboronic acid (450 mg, 3.69 mmol), and cesium carbonate (1.2 g, 3.69 mmol) were suspended in a degassed mixture of dioxane/water (3:1, 12 mL). Pd(dppf)Cl_2 (20 mg, 0.025 mmol) was added, the vial sealed and heated in a microwave at 70 °C for 15 min. The reaction mixture was diluted with ethyl acetate. The organic layer was separated and the aqueous layer extracted with ethyl acetate (10 mL). The organic layers were collected and dried over sodium sulfate, and the solvent was evaporated to dryness. The residue was purified by flash chromatography (biotage SP1, ethyl acetate/hexane, 1/1), yielding 170 mg (68%) of 2-chloro-4-methyl-6-phenylpyrimidine. ^{1}H NMR (401 MHz, DMSO-d_6) δ 8.15−8.21 (m, 2H), 8.06 (s, 1H), 7.52−7.64 (m, 3H), 2.54 (s, 3H). HRMS (ESI) calcd for $C_{11}H_{10}ClN_2$ $[M + H]^+$ 205.0527, found 205.0534.

To a solution of 2-chloro-4-methyl-6-phenylpyrimidine (32 mg, 0.157 mmol) in dioxane (3 mL), 4-mercaptomethylbenzoic acid (53 mg, 0.314 mmol) and DBU (0.07 mL, 0.47 mmol) were added. The final suspension was heated in a microwave at 150 °C for 1 h. The solvent was evaporated to dryness, and the residue was treated with HCl 10% (3 mL) and extracted with DCM (3 × 3 mL). The organic layers were collected and dried over sodium sulfate, and the solvent was evaporated to dryness. The crude was purified by preparative HPLC affording the title compound 4-(4-methyl-6-phenylpyrimidin-2-ylsulfanylmethyl)benzoic acid (13.6 mg, 26%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 8.11−8.18 (m, 2H), 7.84−7.90 (m, 2H), 7.70 (s, 1H), 7.59 (d, J = 8.42 Hz, 2H), 7.50−7.56 (m, 3H), 4.56 (s, 2H), 2.48 (s, 3H). HRMS (ESI) calcd for $C_{19}H_{17}N_2O_2S$ $[M + H]^+$ 337.1005, found 337.1018.

For NMR structural characterization of compound **1**, see Supporting Information, Figure S5, p S34).

By analogous procedure, compounds **2**−**6**, **8**−**10**, and **12**−**20** were prepared from 2,4-dichloro-6-methylpyrimidine using different boronic acids, thiols, and amines. Compound **11** was prepared from 2,4-dichloropyrimidine using phenylboronic acid and 4-aminomethyl-cyclohexanecarboxylic acid (see Scheme 1 and Supporting Information, p S10).

4-(4-Methyl-6-phenylpyrimidine-2-sulfonylmethyl)benzoic Acid (7). To a solution of 4-(4-methyl-6-phenylpyrimidin-2-ylsulfanylmethyl)benzoic acid (**1**) (20 mg, 0.06 mmol) in THF (1 mL), *m*-chloroperoxybenzoic acid (55% in weight) (40 mg, 0.18 mmol) was added, and the final suspension was stirred at room temperature for 3 h. The mixture was diluted with DCM (5 mL) and washed with $NaHCO_3$ sat. (2 × 5 mL). The organic layer was collected and dried over sodium sulfate and the solvent evaporated to dryness. The crude was purified by preparative HPLC affording the title compound 4-(4-methyl-6-phenylpyrimidine-2-sulfonylmethyl)-benzoic acid (6 mg, 27%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 8.32 (s, 1H), 8.24−8.30 (m, 1H), 7.84−7.94 (m, 2H), 7.57−7.65 (m, 3H), 7.51−7.56 (m, 3H), 5.17 (s, 2H), 2.67 (s, 3H). HRMS (ESI) calcd for $C_{19}H_{16}N_2O_4S$ $[M + H]^+$ 369.0904, found 369.0919.

4-[(6-Oxo-4-phenyl-1,6-dihydropyrimidin-2-ylamino)-methyl]cyclohexanecarboxylic Acid ethyl ester (22). In a microwave vial 4-guanidinomethylcyclohexanecarboxylic acid (**21**) (6

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g, 30 mmol) and ethyl benzoylacetate (7.8 mL, 45 mmol) were mixed and heated at 180 °C for 2 h. After cooling, the suspension was filtered, washed twice with diethyl ether, and dried in air flow. The resulting solid was suspended in ethanol (250 mL), treated with sulfuric acid 98% (0.5 mL), and heated at reflux for 1 h. The final solution was evaporated to dryness and the residue taken up with DCM (200 mL) and washed with NH_4Cl sat. (twice) and water. The organic layer was dried over sodium sulfate and the solvent evaporated to dryness affording the title compound 4-[(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester (**22**) (2.15 g, 20%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 0.57 (bs, 1 H), 7.89−8.02 (m, 2 H), 7.36−7.47 (m, 3 H), 6.54 (bs, 1 H), 6.11 (s, 1 H), 4.04 (q, J = 7.2 Hz, 2 H), 2.17−2.29 (m, 1 H), 1.86−1.98 (m, J = 10.5 Hz, 2 H), 1.81 (d, J = 13.3 Hz, 2 H), 1.46−1.61 (m, 1 H), 1.26−1.38 (m, J = 12.7 Hz, 3 H), 1.16 (t, J = 7.1 Hz, 3 H), 0.89−1.12 ppm (m, J = 15.5 Hz, 2 H). HRMS (ESI) calcd for $C_{20}H_{26}N_3O_3$ $[M + H]^+$ 356.1969, found 356.1964.

For NMR structural characterization of compound **22**, see Supporting Information, Figure S7, p S38).

4-[(4-Chloro-6-phenylpyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic Acid Ethyl Ester (23). $POCl_3$ (6.1 mL, 66.54 mmol) and dimethylaniline (0.23 mL, 1.81 mmol) were added to 4-[(6-oxo-4-phenyl-1,6-dihydropyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic acid ethyl ester (**22**) (2.15 g, 6.05 mmol) in a 100 mL bottom-round flask, and the resulting suspension was heated at reflux for 1 h. The purple solution was evaporated to dryness and the residue was redissolved twice in toluene and dried. The crude was suspended in water and extracted twice with diethyl ether. The organic layers were collected and dried over sodium sulfate and the solvent was evaporated to dryness affording the title compound 4-[(4-chloro-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester (**23**) (2.05 g, 90%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 8.08 (bs, 2H), 7.66−7.73 (m, 1H), 7.42−7.56 (m, 3H), 7.19 (s, 1H), 3.95−4.06 (m, 2H), 2.20 (s, 1H), 1.86 (bs, 2H), 1.69−1.82 (m, 2H), 1.52 (bs, 1H), 1.18−1.37 (m, 3H), 1.08−1.18 (m, 3H), 0.90−1.05 (m, 2H). HRMS (ESI) calcd for $C_{20}H_{25}ClN_3O_2$ $[M + H]^+$ 374.1630, found 374.1628.

4-[(4,6-Diphenylpyrimidin-2-ylamino)methyl]cyclohexane-carboxylic Acid (24). In a microwave vial, to a solution of 4-[(4-chloro-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester (**23**) (40 mg, 0.11 mmol) in a predegassed mixture of dioxane/water (3:1, 1.2 mL), Cs_2CO_3 (105 mg, 0.32 mmol), phenylboronic acid (39 mg, 0.32 mmol), and Pd(dppf)Cl_2 (2 mg, 0.0025 mmol) were added. The sealed vial was heated in a microwave at 100 °C for 20 min. The upper phase of the final bilayer system (dioxane) was transferred and dried. The residue was dissolved in DCM and eluted with DCM on a prepacked silica cartridge (0.5 g of silica). The volatiles were removed under reduced pressure, and the residue was dissolved in THF/water/MeOH 2:2:1 (2 mL) and treated with LiOH·H_2O (6.5 mg, 0.15 mmol). The resulting solution was stirred at room temperature for 24 h. Then HCl 1 N (0.15 mL, 0.15 mmol) was added and the mixture was evaporated to dryness. The residue was dissolved in DMSO (1 mL), filtered, and purified by preparative HPLC, affording the title compound 4-[(4,6-diphenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid (**24**) (10 mg, 24%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 11.93 (bs, 1H), 8.23 (bs, 4H), 7.68 (s, 1H), 7.49−7.58 (m, 6H), 7.22−7.31 (m, 1H), 3.32−3.38 (m, 2H), 2.09−2.19 (m, 1H), 1.82−1.96 (m, 4H), 1.53−1.69 (m, 1H), 1.19−1.37 (m, 2H), 0.95−1.12 (m, 2H). HRMS (ESI) calcd for $C_{24}H_{26}N_3O_2$ $[M + H]^+$ 388.2020, found 388.2025.

By analogous procedure, starting from 4-[(4-chloro-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester (**23**) and using the appropriate boronic acid or ester, compounds **25**−**48** were prepared with a 20% average yield (see Scheme 2 and Supporting Information, p S14).

4-[(4-Benzylamino-6-phenylpyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic Acid (49). In a microwave vial, to a solution of 4-[(4-chloro-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester (**23**) (250 mg, 0.69 mmol) in ACN (4.5 mL), benzylamine (0.091 mL, 0.83 mmol) and DIPEA (0.178 mL, 1.04 mmol) were added. The vial was sealed and heated at 180 °C for 1 h. The resulting solution was dried under vacuum and the residue was dissolved in DCM and washed twice with NH_4Cl saturated solution. The organic layer was dried over sodium sulfate, the solvent was evaporated to dryness, and the crude was purified by flash chromatography (DCM/MeOH 98:2), yielding 192 mg (67%) of 4-[(4-benzylamino-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester. ^{1}H NMR (401 MHz, DMSO-d_6) δ 7.88 (bs, 2H), 7.38−7.48 (m, 4H), 7.27−7.36 (m, 4H), 7.18−7.25 (m, 1H), 6.53 (bs, 1H), 6.25 (bs, 1H), 4.50−4.55 (m, 2H), 3.96−4.07 (m, 2H), 3.12 (bs, 2H), 2.21 (bs, 1H), 1.85 (bs, 4H), 1.49 (bs, 1H), 1.23 (bs, 2H), 1.13 (t, J = 7.14 Hz, 3H), 0.91 (bs, 2H). The 4-[(4-benzylamino-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester thus obtained was dissolved in THF/water/MeOH 2:2:1 (2.4 mL) and treated with LiOH·H_2O (37.8 mg, 0.90 mmol). The solution was stirred at room temperature for 18 h. Then HCl 1 N (0.9 mL, 0.9 mmol) was added and organic volatiles were removed under reduced pressure. The resulting suspension was extracted twice with DCM. The organic layer was dried over sodium sulfate and the solvent evaporated to dryness, providing the title compound 4-[(4-benzylamino-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid (**49**) (120 mg, 64%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 12.00 (bs, 1H), 9.39 (bs, 1H), 7.79 (bs, 3H), 7.55−7.70 (m, 3H), 7.32−7.43 (m, 4H), 7.22−7.32 (m, 1H), 6.41 (s, 1H), 4.60−4.69 (m, 2H), 3.21−3.28 (m, 2H), 2.08 (d, J = 9.52 Hz, 1H), 1.85 (bs, 2H), 1.69 (bs, 2H), 1.45 (bs, 1H), 1.11−1.30 (m, 2H), 0.84−1.01 (m, 2H). HRMS (ESI) calcd for $C_{25}H_{29}N_4O_2$ $[M + H]^+$ 417.2285, found 417.2289.

By analogous procedure, starting from 4-[(4-chloro-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester (**23**) and using the appropriate amine, compounds **51** and **52** were prepared with a 65% average yield (see Scheme 2 and Supporting Information, p S20).

4-[(4-Benzylamino-6-phenylpyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic Acid Amide (53). 4-[(4-Benzylamino-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid (**49**) (40 mg, 0.1 mmol) was suspended in dry DCM (1.5 mL), and EDC·HCl (29 mg, 0.15 mmol), NH_4OBt (23 mg, 0.15 mmol) and DIPEA (0.035 mL, 0.2 mmol) were added. The resulting suspension was stirred at room temperature for 6 h, diluted with DCM (5 mL), and washed twice with NH_4Cl saturated solution and then with $NaHCO_3$ saturated solution. The organic layer was dried over sodium sulfate, the solvent evaporated to dryness, and the crude purified by preparative HPLC, affording the title compound 4-[(4-benzylamino-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid amide (**53**) (25 mg, 60%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 11.79 (bs, 1H), 9.36 (bs, 1H), 7.73 (d, J = 6.35 Hz, 2H), 7.56−7.66 (m, 3H), 7.41 (bs, 1H), 7.32−7.39 (m, 4H), 7.25−7.32 (m, 1H), 7.14 (bs, 1H), 6.64 (bs, 1H), 6.38 (s, 1H), 4.64 (d, J = 5.61 Hz, 2H), 2.00 (t, J = 11.84 Hz, 1H), 1.63−1.81 (m, 4H), 1.47 (bs, 1H), 1.18−1.33 (m, 2H), 0.78−0.96 (m, 2H). HRMS (ESI) calcd for $C_{25}H_{30}N_5O$ $[M + H]^+$ 416.2445, found 416.2431.

By analogous procedure, starting from 4-[(4-benzylamino-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid (**49**) and using the appropriate nucleophile, compounds **54**−**57** were prepared with a 60% average yield (see Scheme 2 and Supporting Information, p S21).

4-[(4-Chloro-6-phenylpyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic Acid Amide (58). 4-[(4-Chloro-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester (**23**) (3.62 g, 10.06 mmol) was dissolved in THF/water/MeOH 2:2:1 (50 mL) and treated with LiOH·H_2O (1.28 g, 30.18 mmol). The solution was stirred at room temperature for 18 h. Then HCl 1 N (30 mL, 30 mmol) was added and organic volatiles were removed under reduced pressure. The resulting suspension was extracted twice with DCM. The organic layer was dried over sodium sulfate and the solvent evaporated to dryness to give 4-[(4-chloro-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid (2.97 g, 8.59 mmol). The crude compound, without further purification, was suspended in a mixture of DCM/DMF 10:1 (100 mL) and treated with EDC·HCl

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(2.47 g, 12.88 mmol), NH_4OBt (1.96 g, 12.88 mmol), and DIPEA (2.94 mL, 17.18 mmol). The suspension was stirred at room temperature for 16 h, diluted with DCM (150 mL), and washed twice with NH_4Cl saturated solution, then with $NaHCO_3$ saturated solution. The solid in the organic layer was collected by filtration to give the title compound 4-[(4-chloro-6-phenylpyrimidin-2-ylamino)-methyl]cyclohexanecarboxylic acid amide (**58**) (2.1 g, 60%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 8.12 (d, J = 4.64 Hz, 2H), 7.72 (t, J = 5.74 Hz, 1H), 7.47−7.57 (m, 3H), 7.22 (s, 1H), 7.11 (bs, 1H), 6.61 (bs, 1H), 3.24−3.32 (m, 2H)1.96−2.09 (m, 1H), 1.70−1.87 (m, 4H), 1.55 (bs, 1H), 1.22−1.37 (m, 2H), 0.97 (d, J = 7.45 Hz, 2H). HRMS (ESI) calcd for $C_{18}H_{22}ClN_4O$ $[M + H]^+$ 345.1477, found 345.1481.

4-{[4-(2-Methylbenzylamino)-6-phenylpyrimidin-2-ylamino]methyl}cyclohexanecarboxylic Acid Amide (59). In a microwave vial, to a solution of 4-[(4-chloro-6-phenylpyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid amide (**58**) (30 mg, 0.09 mmol) in NMP (1 mL), 2-methylbenzylamine (0.021 mL, 0.17 mmol) and DIPEA (0.087 mL, 0.51 mmol) were added, and the sealed vial was heated at 220 °C for 15 min. The final solution was directly purified by preparative HPLC, affording the title compound 4-{[4-(2-methylbenzylamino)-6-phenylpyrimidin-2-ylamino]methyl}-cyclohexanecarboxylic acid amide (**59**) (22.3 mg, 58%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 7.80−7.97 (m, 2H), 7.36−7.47 (m, 3H), 7.24−7.30 (m, 1H), 6.97−7.21 (m, 4H), 6.59 (bs, 2H), 6.50 (bs, 1H), 6.28 (bs, 1H), 4.45−4.54 (m, 2H), 3.04−3.15 (m, 2H), 2.33 (s, 3H), 1.99 (t, J = 12.14 Hz, 1H), 1.60−1.85 (m, J = 12.69 Hz, 4H), 1.48 (bs, 1H), 1.10−1.36 (m, J = 10.86 Hz, 2H), 0.70−0.98 (m, 2H). HRMS (ESI) calcd for $C_{26}H_{32}N_5O$ $[M + H]^+$ 430.2601, found 430.2597.

By analogous procedure, starting from 4-[(4-chloro-6-phenyl-pyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid amide (**58**) and using the appropriate benzylamine, compounds **60**−**75** were prepared with a 40% average yield (see Scheme 2 and Supporting Information, p S23).

Benzyl-(6-chloro-2-methylsulfanylpyrimidin-4-yl)amine (77). A solution of 4,6-dichloro-2-methylsulfanylpyrimidine (**76**) (1 g, 5.13 mmol) in EtOH (10 mL) was treated with benzylamine (0.615 mL, 5.64 mmol) and DIPEA (1.75 mL, 10.25 mmol) and heated at 150 °C in a sealed microwave vial for 30 min. The resulting mixture was dried, dissolved in DCM (50 mL), and washed twice with NH_4Cl saturated solution. The organic layer was collected and dried over sodium sulfate and the solvent evaporated to dryness affording the title compound benzyl-(6-chloro-2-methylsulfanylpyrimidin-4-yl)amine (**77**) (1.24 g, 90%). ^{1}H NMR (401 MHz, DMSO-d_6) δ 8.21−8.32 (m, 1H), 7.29−7.39 (m, 4H), 7.23−7.27 (m, 1H), 6.28 (s, 1H), 4.50−4.59 (m, 2H), 2.39 (s, 3H). HRMS (ESI) calcd for $C_{12}H_{13}ClN_3S$ $[M + H]^+$ 266.0513, found 266.0514.

(6-Chloro-2-methylsulfanylpyrimidin-4-yl)phenylamine (78). A solution of 4,6-dichloro-2-methylsulfanylpyrimidine (**76**) (500 mg, 2.56 mmol) in EtOH (5 mL) was treated with aniline (0.257 mL, 2.82 mmol) and DIPEA (0.6 mL, 3.5 mmol) and heated at 120 °C in a sealed microwave vial for 2 h with the cooling function activated. The resulting mixture was dried, dissolved in DCM (50 mL), and washed twice with NH_4Cl saturated solution. The organic layer was collected and dried over sodium sulfate. The residue was purified by flash-chromatography (DCM), yielding 558 mg (87%) of the title compound (6-chloro-2-methylsulfanylpyrimidin-4-yl)phenylamine (**78**). ^{1}H NMR (401 MHz, DMSO-d_6) δ 9.87 (s, 1H), 7.59 (d, J = 7.93 Hz, 2H), 7.36 (t, J = 7.93 Hz, 2H), 7.08 (t, J = 7.38 Hz, 1H), 6.49 (s, 1H), 2.48 (s, 3H). HRMS (ESI) calcd for $C_{11}H_{11}ClN_3S$ $[M + H]^+$ 252.0357, found 252.0359.

4-[(4-Benzylamino-6-chloropyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic Acid Ethyl Ester (79). To a solution of benzyl-(6-chloro-2-methylsulfanylpyrimidin-4-yl)amine (**77**) (1.1 g, 4.14 mmol) in DCM (42 mL), *m*-chloroperoxybenzoic acid (55% in weight) (2.66 g, 8.48 mmol) was added, and the resulting suspension was stirred at room temperature for 20 h. The mixture was washed with $NaHCO_3$ saturated solution (3 × 50 mL) and the organic layers were collected, dried over sodium sulfate, and evaporated to dryness, affording benzyl-(6-chloro-2-methanesulfonylpyrimidin-4-yl)-amine (1.12 g, 91%), that was used without further purification. To a solution of benzyl-(6-chloro-2-methanesulfonylpyrimidin-4-yl)amine (0.5 g, 1.68 mmol) in NMP (17 mL), 4-aminomethylcyclohexane-carboxylic acid ethyl ester (933 mg, 5.04 mmol) and DIPEA (1.15 mL, 6.72 mmol) were added, and the resulting mixture was heated at 180 °C in a sealed microwave vial for 30 min. The mixture was then diluted with AcOEt (50 mL) and washed with NH_4Cl saturated solution. The organic layers were collected, dried over sodium sulfate, and evaporated to dryness. The residue was purified by flash chromatography (hexane/AcOEt 8:2), yielding 1.1 g (27%) of 4-[(4-benzylamino-6-chloropyrimidin-2-ylamino)methyl]cyclohexane-carboxylic acid ethyl ester (**79**). ^{1}H NMR (401 MHz, DMSO-d_6) δ 7.51−7.81 (m, 1H), 7.16−7.40 (m, 5H), 6.77−7.10 (m, 1H), 5.59−5.86 (m, 1H), 4.35−4.58 (m, 1H), 3.91−4.12 (m, 2H), 2.93−3.07 (m, 2H), 2.03−2.27 (m, 1H), 1.58−1.92 (m, 2H), 1.30−1.51 (m, 1H), 1.04−1.30 (m, 5H), 0.69−1.00 (m, 2H). HRMS (ESI) calcd for $C_{21}H_{28}ClN_4O_2$ $[M + H]^+$ 403.1896, found 403.1885.

4-[(4-Chloro-6-phenylaminopyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic Acid Ethyl Ester (80). To a solution of (6-chloro-2-methylsulfanylpyrimidin-4-yl)phenylamine (**78**) (100 mg, 0.40 mmol) in DCM (4 mL), *m*-chloroperoxybenzoic acid (55% in weight) (257 mg, 0.82 mmol) was added, and the resulting suspension was stirred at room temperature for 20 h. The mixture was washed with $NaHCO_3$ saturated solution (3 × 50 mL), and the organic layers were collected, dried over sodium sulfate, and evaporated to dryness affording (6-chloro-2-methanesulfonylpyrimidin-4-yl)phenylamine (101 mg, 90%) that was used without further purification. To a solution of (6-chloro-2-methanesulfonylpyrimidin-4-yl)phenylamine (101 mg, 0.36 mmol) in NMP (1.8 mL), 4-aminomethyl-cyclohexane-carboxylic acid ethyl ester (200 mg, 1.08 mmol) and DIPEA (0.25 mL, 1.44 mmol) were added, and the resulting mixture was heated at 180 °C in a sealed microwave vial for 30 min. The final solution was diluted with AcOEt (10 mL) and washed with NH_4Cl saturated solution. The organic layers were collected, dried over sodium sulfate, and evaporated to dryness. The residue was purified by flash chromatography (hexane/AcOEt 8:2) yielding 23 mg (15%) of the title compound 4-[(4-chloro-6-phenylaminopyrimidin-2-ylamino)-methyl]cyclohexanecarboxylic acid ethyl ester (**80**). ^{1}H NMR (401 MHz, DMSO-d_6) δ 8.02 (bs, 1H), 7.85 (d, J = 7.57 Hz, 2H), 7.75 (bs, 2H), 6.25 (s, 1H), 3.95−4.12 (m, 2H), 2.94−3.07 (m, 2H), 2.03−2.28 (m, 1H), 1.58−1.90 (m, 2H), 1.32−1.51 (m, 1H), 1.04−1.32 (m, 5H), 0.68−1.05 (m, 2H). HRMS (ESI) calcd for $C_{20}H_{26}ClN_4O_2$ $[M + H]^+$ 389.1739, found 389.1742.

4-[(4-Phenyl-6-phenylaminopyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic Acid (50). In a microwave vial, to a solution of 4-[(4-chloro-6-phenylaminopyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic acid ethyl ester (**80**) (20 mg, 0.05 mmol 0.11 mmol) in a predegassed mixture of dioxane/water (3:1, 0.6 mL), Cs_2CO_3 (50 mg, 0.16 mmol), phenylboronic acid (20 mg, 0.16 mmol), and Pd(dppf)Cl_2 (1 mg, 0.0012 mmol) were added. The sealed vial was heated in a microwave at 100 °C for 20 min. The upper phase of the final bilayer system (dioxane) was transferred and dried. The residue was dissolved in DCM and eluted with DCM on a prepacked silica cartridge (0.5 g of silica). The volatiles were removed under reduced pressure, and the residue was dissolved in THF/water/MeOH 2:2:1 (2 mL) and treated with LiOH·H_2O (3.2 mg, 0.07 mmol). The resulting mixture was stirred at room temperature for 24 h, treated with HCl 1 N (0.07 mL, 0.07 mmol), and evaporated to dryness. The residue was dissolved in DMSO (1 mL), filtered, and purified by preparative HPLC, affording the title compound 4-[(4-phenyl-6-phenylaminopyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid (**50**) (5 mg, 24%).^{1}H NMR (401 MHz, DMSO-d_6) δ 10.43 (bs, 1H), 8.08 (bs, 1H), 7.86 (d, J = 7.57 Hz, 2H), 7.75 (bs, 2H), 7.65 (bs, 3H), 7.42 (t, J = 7.45 Hz, 2H), 7.19 (bs, 1H), 6.60 (bs, 1H), 2.08−2.21 (m, 1H), 1.94 (d, J = 10.25 Hz, 2H), 1.83 (d, J = 11.11 Hz, 2H), 1.64 (bs, 1H), 1.17−1.36 (m, 2H), 0.94−1.13 (m, 2H). HRMS (ESI) calcd for $C_{24}H_{27}N_4O_2$ $[M + H]^+$ 403.2129, found 403.2140.

4-[(4-Benzylamino-6-chloropyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic Acid Amide (81). 4-[(4-Benzylamino-6-chloropyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid ethyl ester (**79**) (1.4 g, 3.47 mmol) was dissolved in THF/water/MeOH

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2:2:1 (35 mL) and treated with LiOH·H_2O (437 mg, 10.42 mmol). The mixture was stirred at room temperature for 18 h. Then HCl 1 N (10 mL, 10 mmol) was added and organic volatiles were removed under reduced pressure. The resulting suspension was filtered, washed with water and twice with diethyl ether, and dried in air flow to give 4-[(4-benzylamino-6-chloropyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic acid (1.24 g, 3.29 mmol, 95% yield). The crude material was then suspended in DCM/DMF 10:1 (32 mL) and treated with EDC·HCl (0.947 g, 4.94 mmol), NH_4OBt (0.751 g, 4.94 mmol), and DIPEA (1.13 mL, 6.59 mmol). The resulting suspension was stirred at room temperature for 16 h, then diluted with DCM (50 mL) and washed twice with NH_4Cl saturated solution with $NaHCO_3$ saturated solution. The organic layers were collected, dried over sodium sulfate, and evaporated to dryness. The residue was purified by flash chromatography (DCM/MeOH 98:2) yielding 1.0 g (81%) of 4-[(4-benzylamino-6-chloropyrimidin-2-ylamino)methyl]-cyclohexanecarboxylic acid amide (**81**). ^{1}H NMR (401 MHz, DMSO-d_6) δ 7.71 (bs, 1H), 7.18−7.37 (m, 5H), 7.11 (bs, 1H), 7.00 (bs, 1H), 6.59 (bs, 1H), 5.74 (d, J = 3.30 Hz, 1H), 4.39−4.55 (m, 2H), 3.00 (t, J = 6.29 Hz, 2H), 1.97 (bs, 1H), 1.69 (bs, 4H), 1.39 (bs, 1H), 1.23 (bs, 2H), 0.59−0.99 (m, 2H). HRMS (ESI) calcd for $C_{19}H_{25}ClN_5O$ $[M + H]^+$ 374.1742, found 374.1760.

4-{[4-Benzylamino-6-(2-fluorophenyl)pyrimidin-2-ylamino]-methyl}cyclohexanecarboxylic Acid Amide (82). In a microwave vial, to a solution of 4-[(4-benzylamino-6-chloropyrimidin-2-ylamino)-methyl]cyclohexanecarboxylic acid amide (**81**) (25 mg, 0.07 mmol) in a predegassed mixture of dioxane/water (3:1, 1.2 mL), Cs_2CO_3 (68.5 mg, 0.32 mmol), 2-fluorophenylboronic acid (29.4 mg, 0.21 mmol), and Pd(dppf)Cl_2 (2 mg, 0.0025 mmol) were added. The sealed vial was heated in a microwave at 100 °C for 30 min. The upper phase of the final bilayer system (dioxane) was transferred and dried. The residue was dissolved in AcOEt and eluted with AcOEt/MeOH on a prepacked PL-SH cartridge. After removal of the volatiles under reduced pressure, the residue was dissolved in DMSO (1 mL), filtered and purified by preparative HPLC to give the title compound 4-{[4-benzylamino-6-(2-fluorophenyl)pyrimidin-2-ylamino]methyl}-cyclohexanecarboxylic acid amide (**82**) (4 mg, 13%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.90 (bs, 1H), 7.41−7.71 (m, 4H), 7.20−7.37 (m, 7H), 7.14 (bs, 1H), 6.63 (bs, 1H), 6.42−6.58 (m, 0H), 6.07−6.35 (m, 1H), 4.45−4.58 (m, 2H), 1.99 (bs, 1H), 1.72 (d, J = 11.53 Hz, 4H), 1.35−1.58 (m, 1H), 1.14−1.32 (m, 2H), 0.80 (m, 1H), 0.85 (bs, 2H). HRMS (ESI) calcd for $C_{25}H_{29}FN_5O$ $[M + H]^+$ 434.2351, found 434.2352.

By analogous procedure, starting from 4-[(4-benzylamino-6-chloropyrimidin-2-ylamino)methyl]cyclohexanecarboxylic acid amide (**81**) and using the appropriate boronic acids or esters, compounds **83**−**102** were prepared (see Scheme 3 and Supporting Information, p S28).

2. Protein Expression and Purification. Human full length VCP (residues 2−806) was expressed in High5 insect cells as His-Gst tagged proteins, using baculovirus expression vector based on pVL1393 (Invitrogen). Cells were lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10% glycerol, 0.2% CHAPS, 20 mM DTT, and protease inhibitors), and the cleared lysates were loaded on a glutathione Sepharose 4B (Amersham Biosciences) or nickel Sepharose column. N-Terminal tags were removed by on-column cleavage by addition of PreScission protease (Amersham Biosciences), and the resulting cleaved recombinant proteins were eluted in a final buffer containing 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA. Size exclusion chromatography (Superdex200 16/60 column) and NativePAGE Novex gel were used to analyze VCP proteins oligomerization status.

3. Biochemical Assay. The ATPase activity of recombinant VCP wt was evaluated by monitoring ADP formation, using a modified version of NADH coupled assay.[18] Because ADP and NADH are two ATP competitive inhibitors of VCP ATPase activity, the standard protocol of NADH coupled assay was splitted into a two-step procedure. In the first step, an ATP regenerating system (40 U/mL pyruvate kinase and 3 mM phosphoenol pyruvate by Sigma-Aldrich) converted the ADP produced by VCP activity back to ATP, keeping constant the substrate concentration and preventing product inhibition. In the second step, after quenching VCP enzymatic reaction with 30 mM EDTA and 250 μM NADH, the stoichiometric amount of pyruvate produced during the previous step was reduced by 40 U/mL lactic dehydrogenase (Sigma-Aldrich), resulting in the oxidation of an equivalent amount of NADH. The decrease of NADH concentration was then measured at 340 nm using Tecan Safire 2 reader plate. Compounds were assayed in 96- or 384-well UV plates (Corning) using a reaction buffer containing 50 mM Hepes at pH 7.5, 0.2 mg/mL BSA, 10 mM $MgCl_2$, and 2 mM DTT. After 20 min of preincubation with 150 nM VCP, 60 μM ATP was added to the reaction mixture that was then allowed to proceed for 90 min before quenching.

4. Cell Culture. HCT-116 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). HCT-116 cells were cultured in McCoy's 5A medium (Gibco BRL, Gaithersburg, MD, USA) at 37 °C in a humidified atmosphere with 5% CO_2. Media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco BRL), 100 units/mL penicillin, and 100 μg/mL streptomycin.

5. Inhibition of Cell Proliferation. Cells were seeded at 1600/well in 384-well white clear-bottom plates (Greiner). At 24 h after seeding, cells were treated with the compounds to be tested and incubated for an additional 72 h at 37 °C under a 5% CO_2 atm. At the end of incubation, cells were lysed and the ATP content in the well was used as a measure of viable cells. This was determined using a thermostable firefly luciferase based assay (CellTiter-Glo) from Promega. IC_{50} values were calculated using the percentage of growth versus the untreated control.

6. Assessment of the Mechanism of Action of the Compounds. First, 1.2−1.5 million of exponentially growing HCT-116 cells were seeded in 35 mm plates 24 h before treatment. Then an amount of 3 μL of compound diluted in DMSO was added to 3 mL of the appropriate medium in the well with 0.1% DMSO final concentration, and the plates were incubated for 8 h. Cells were washed twice with ice cold PBS, lysed in RIPA buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, protease inhibitors cocktail (Sigma P8340), phosphatase inhibitors cocktails I and II (Sigma P2850 and P5725), and the lysates clarified by centrifugation for 10 min at 16000g. Then an amount of 20 μg of each sample was fractionated in 4−12% SDS−PAGE and blotted onto a nitrocellulose transfer membrane (Whatman-Protan nitrocellulose transfer membrane 10401196). Immunoblot was performed with the described antibodies in TBS 1× (Biorad) with 5% milk and 0.1% Tween 80. HRP-conjugated secondary antibodies were used 1:10000 (Immunopure goat anti-mouse and Immunopure goat anti-rabbit from Thermo-Scientific). The detection was performed using SuperSignal West Pico Chemiluminescent substrate from Thermo Scientific.

7. Molecular Modeling. Ab initio geometry optimization of representative compounds was carried out with Gaussian 03 program (http://www.gaussian.com)[19] using the density functional theory (DFT) method, the B3LYP exchange and correlation functional with 6-31G* basis set. Molden[20] graphical user interface was used to analyze the results.

Molecular docking of compound **49** in VCP/D2 binding site was performed using QXP/FLO[21] program setting 2500 Monte Carlo conformational search steps on the flexible ligand and saving 10 final poses. VCP/D2 binding site was obtained by the published 3CF0[4] X-ray complex at 3.00 Å resolution. Chain A containing one cocrystallized ADP molecule was selected as representative. Crystallographic water molecules were removed, and all polar hydrogen atoms were added and energy minimized with the suitable QXP/FLO tool. PyMOL 1.3 (http://www.pymol.org) program was used to obtain Figures 2 and 3.

■ ASSOCIATED CONTENT

Supporting Information

Analytical data for additional compounds, biological data, calculated data, and molecular formula strings in csv format. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

VCP, valosine containing protein; cdc48, cell division cycle 48; AAA, ATPases associated with various cellular activities; HTS, high-throughput screening; HCT-116, human colorectal carcinoma 116; C/EBP, CCAAT-enhancer-binding protein; CHOP, CCAAT/enhancer-binding protein homologous protein; Gadd153, growth arrest and DNA damage induced gene 153; NSF, *N*-ethylmaleimide sensitive fusion protein; SPATA5, spermatogenesis-associated protein 5; VPS4B, vacuolar protein sorting-associated protein 4B; Hsp90, heat shock protein 90; LE, ligand efficiency; LLE, lipophilic ligand efficiency

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