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Alkylsulfanyl-1,2,4-triazoles, a New Class of Allosteric Valosine Containing Protein Inhibitors. Synthesis and Structure−Activity Relationships

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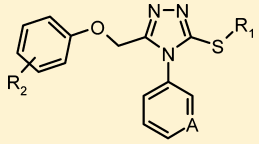
Supporting Information

ABSTRACT: Valosine containing protein (VCP), also known as p97, is a member of AAA ATPase family that is involved in several biological processes and plays a central role in the ubiquitin-mediated degradation of misfolded proteins. VCP is an ubiquitously expressed, highly abundant protein and has been found overexpressed in many tumor types, sometimes associated with poor prognosis. In this respect, VCP has recently received a great deal of attention as a potential new target for cancer therapy. In this paper, the discovery and structure−activity relationships of alkylsulfanyl-1,2,4-triazoles, a new class of potent, allosteric VCP inhibitors, are described. Medicinal chemistry manipulation of compound **1**, identified via HTS, led to the discovery of potent and selective inhibitors with submicromolar activity in cells and clear mechanism of action at consistent doses. This represents a first step toward a new class of potential anticancer agents.



A = C, N

INTRODUCTION

Valosine-containing protein (VCP), also known as p97 (cdc48 in yeast), is a member of the type II ATPases associated with various cellular activities (AAA) family of proteins, which are characterized by the presence of two conserved ATPase domains, also called AAA domains.[1] As for other AAA proteins, VCP is an enzymatic machine that transforms the chemical energy produced by hydrolysis of ATP into mechanical work necessary for a range of cellular processes including ubiquitin-proteasome (Ub-Pr) mediated protein degradation, membrane fusion, transcription activation, cell cycle control, apoptosis, and general chaperone activity.[2] Under physiologic conditions, VCP forms a ring shaped homohexamer, with each protomer containing an *N*-terminal domain (*N*) and two AAA domains designated D1 and D2. The *N* domain has been shown to interact with a variety of effector molecules. The D1 domain mediates hexamerization and has very low hydrolytic activity, while the D2 domain contributes to most of the ATPase activity and it is thought to function as a mechanochemical transducer. In the hexameric structure, D1 and D2 domains form two stacked rings around a central pore with the *N* domains protruding outward from the D1 domains. The VCP hexamer is a highly flexible complex which undergoes global conformational changes during ATP binding and hydrolysis cycles as shown by comparison of the crystal structure reconstructions of representative ATP hydrolysis states.[3,4]

VCP is an essential and highly abundant protein, accounting for more than 1% of total cellular protein content. Because of its involvement in a wide variety of functions, altered expression or mutation of VCP is expected to have pathological consequences. Accordingly, genetic ablation of VCP in mice was found to be embryonic lethal,[5] while mutations in the VCP gene are associated with neurodegenerative diseases.[6] On the other hand, elevated levels of VCP have been detected in many cancer types, sometimes associated with poor prognosis.[7,8] In this respect, VCP has attracted a great deal of attention as a potential new molecular target for cancer therapy. While VCP involvement in a wide spectrum of cellular functions might raise concerns regarding the tolerability of such therapy, it should be noted that germline mutations in the human VCP gene are associated with neurodegenerative diseases that are however of late onset, suggesting that VCP inhibition might be sustainable for a considerable time before inducing irreversible adverse effects.

Although inhibitors interfering with VCP function have been previously described,[9−11] biochemically potent, reversible, and specific compounds with cellular activity and mechanism of action clearly directly related to VCP inhibition have never been reported.

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Here we describe the discovery and structure−activity relationships (SAR) of a series of alkylsulfanyl-1,2,4-triazoles representing a new class of VCP inhibitors, which were identified through biochemical screening of a collection of more than one million compounds and which inhibit the enzyme through an allosteric mechanism not previously described for this enzyme. The original hit of this class (**1**, Figure 1) was able to inhibit VCP ATPase activity with an IC_{50} of 2.69 μM but was devoid of antiproliferative effects up to a concentration of 20 μM. The biochemical potency of **1** was unaffected by altering ATP concentration in assay conditions (Supporting Information Table S1, p S26), suggesting a mechanism of noncompetitive inhibition with respect to this substrate. Moreover, serial dilution experiments and mass spectrometric analysis of VCP upon incubation with molar excess of the inhibitor indicated that it possesses reversible binding properties (data not shown).

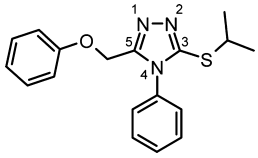

Figure 1. Structure of alkylsulfanyltriazole **1**.

Although more extensive biochemical and biological characterization will be described separately (Magnaghi et al., manuscript submitted), it is nevertheless worth noting within the scope of this report that the putative binding site of the compounds described herein has been identified by photo affinity labeling (PAL) experiments and is located between the D1 and D2 domains of adjacent protomers of the VCP homohexamer in the lateral tunnel leading to the central pore (Figure 2). On this basis, we currently hypothesize that interference with the profound conformational changes which this region undergoes during ATP binding and hydrolysis cycles[12,13] and a resulting impairment of the VCP molecular machinery underlies the allosteric mechanism of this class of inhibitors.

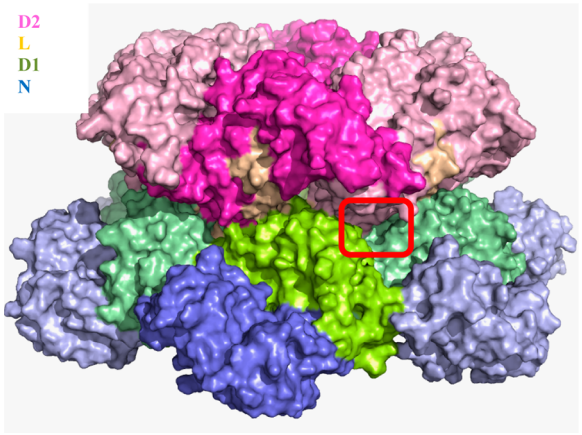

Figure 2. A surface representation of the VCP homohexamer. The approximate region of binding as indicated by photo affinity labeling experiments is highlighted (red boundary). A blue/green/red color scheme is used to distinguish the different VCP domains (see inset for color code).

Starting from compound **1**, a medicinal chemistry program was initiated aimed at improving the biochemical and biological profile of the class. Given the low resolution of available structural data for VCP, attempts to dock compounds into the site identified by PAL experiments did not yield a binding model able to provide a sufficient rationalization of biochemical results observed for initial analogues. This, together with the difficulty encountered in attempts to obtain cocrystals suitable for X-ray diffraction, led us to undertake medicinal chemistry optimization through a traditional iterative SAR approach.

The synthesis of several analogues of **1**, sequentially modified at positions 3, 4, and 5,[14] allowed us to optimize the key features contributing to VCP inhibition and to obtain potent derivatives with submicromolar antiproliferative activity and with a dose-dependent mechanism of action.

Chemistry. Our strategy for the formation of the substituted 4*H*-[1,2,4]triazole-3-thiol ring (general structure **C**, Scheme 1) was based on the cyclization of intermediates **B** in basic media, as reported in the literature.[15]

Intermediates **B** are easily accessible from hydrazides **A** and the suitably substituted isothiocyanate. Compounds **1**−**9**, **11**, **12**, **23**−**45**, **48**−**51**, and **53**−**57** were obtained by *S*-alkylation directly from intermediates **C**, while compound **19** was obtained upon oxidative cleavage with hydrogen peroxide and acetic acid of the corresponding intermediate **18**.[16] Compounds **1** and **2** were then subjected to *S*-oxidation by reaction with 3-chloroperbenzoic acid to provide the sufonyl derivatives **16** and **17**.[17] Compound **16** was in turn transformed into compounds **10** and **20**−**22** by nucleophilic displacement of the methylsulfone with thiophenol, 2-propanol, cyclopentanol, and cyclohexanol respectively.[18] Compound **46** was obtained upon treatment of **39** with iron and ammonium chloride in methanol/water at reflux temperature.[19] Compound **46** was then converted to sodium azide **47** via diazonium salt.

Treatment of compounds **12** and **50** with boron trichloride gave the hydroxyl derivatives **13** and **59** that were converted to the activated chloro analogues **14** and **15** useful for the left-hand expansion of the scaffold. Product **15** was transformed into the *N*-derivatives **52** and **58** by reaction with the corresponding aniline, while the *O*-derivatives **60** and **66** were obtained by treatment of **15** respectively with cyclohexanol and 3-hydroxypyridine.[20]

N-Isosters **63**−**65** (Scheme 2) were obtained by cyclization of methyl isothioureas **E** with phenoxyacetic acid hydrazide[21] and subsequent removal of the benzylic protection of intermediates **F**. Isothioureas **E** could be prepared by methylation of the corresponding thioureas **D** readily obtained from phenyl isothiocyanate and the appropriate benzylalkylamine.[22]

C-Isosters **61** and **62** were synthesized similarly to previously reported protocols,[23] as depicted in Scheme 3. Amides **G**, obtained by conventional methods, were treated with phosphorus pentasulfide to give thioamides **H**. *S*-Alkylation with methyl iodide yielded the alkyl thioimidate derivatives **I**, which were subjected to cyclization to the final compounds **61** and **62** by heating with phenoxyacetic acid hydrazide.

Variations at the left-hand part of the scaffold were achieved as described in Scheme 4. Triazoles **67**−**90** were obtained by treatment of the chloro intermediate **14** with the suitable substituted phenols **J** and potassium carbonate in dimethylformamide at 80−100 °C.[24] Compounds **94**−**118** were synthesized by Suzuki coupling, under microwave irradiation, of derivatives **75** or **83** with the appropriate aryl boronic acids

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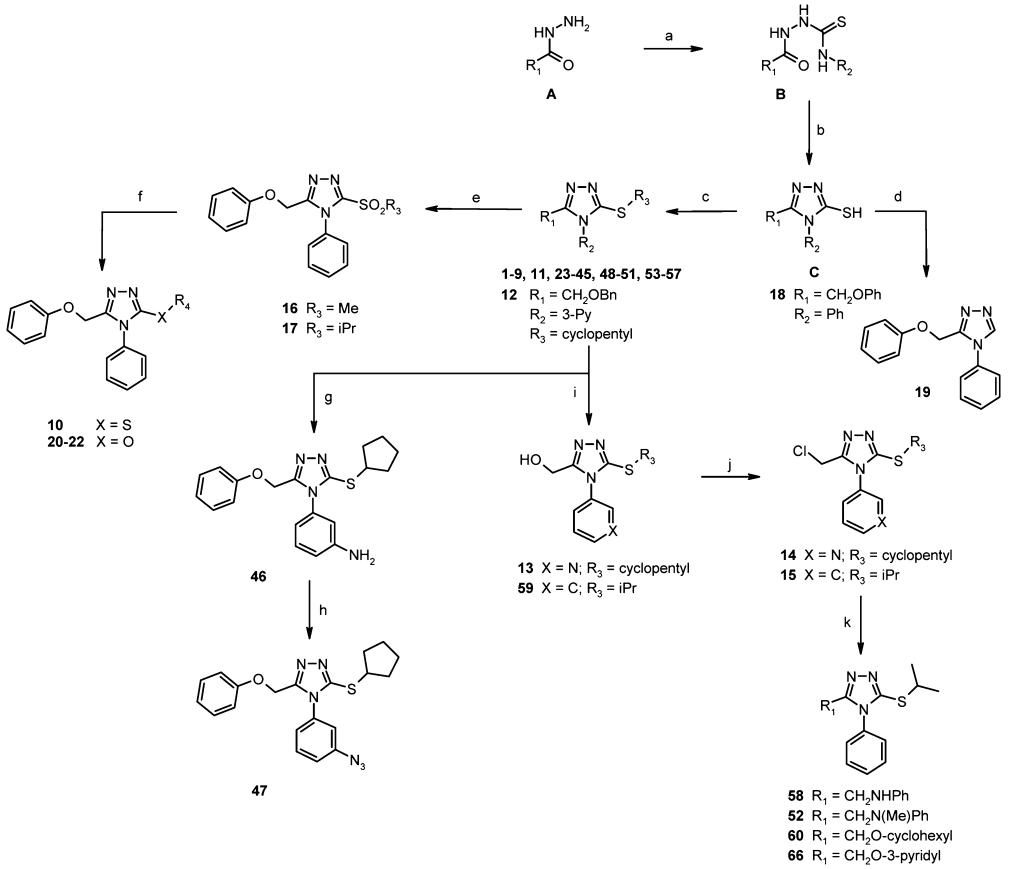
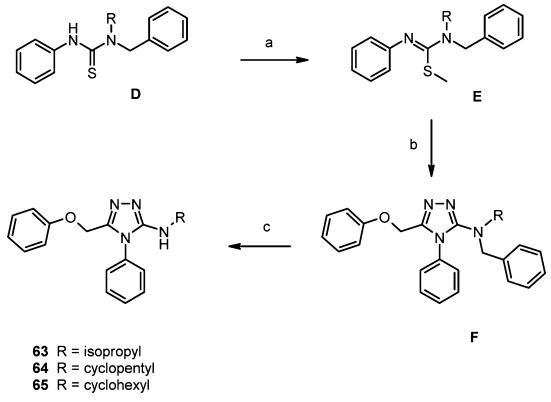
Scheme 1. General Synthesis of 3-Alkylsulfanyl and 3-Alkoxy-1,2,4-triazoles[a]



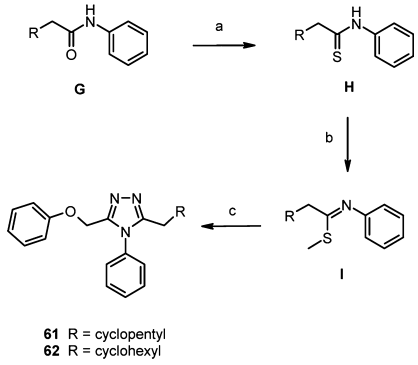
[a]Reagents and conditions: (a) R_2NCS, EtOH, reflux; (b) 1 M NaOH, reflux; (c) R_3Br or R_3I, K_2CO_3, DMF, rt; (d) from **18**, H_2O_2, acetic acid, CH_2Cl_2, rt; (e) from **1** or **2**, 3-chloroperbenzoic acid, CH_2Cl_2, rt; (f) from **16**, R_4OH or R_4SH, 60% NaH, THF, rt; (g) from **39**, Fe, NH_4Cl, MeOH/H_2O, reflux; (h) $NaNO_3$, NaN_3, 20% HCl, 0−5 °C; (i) from **12**or **50**, 1.0 M BCl_3, CH_2Cl_2, 0−5 °C; (j) $SOCl_2$, CH_2Cl_2, 0−5 °C; (k) from **15**, for **58** and **52**, appropriate aniline, K_2CO_3, DMF, 80 °C, for **60** and **66**, cyclohexanol or 3-hydroxypyridine, 60% NaH, THF, reflux.

Scheme 2. Synthesis of 3-Alkylamino-1,2,4-triazoles[a]



[a]Reagents and conditions: (a) MeI, *t*-BuOK, THF, 0−5 °C; (b) phenoxyacetic hydrazide, TFA, THF, reflux; (c) formic acid, 5% Pd/C, 80 °C.

Scheme 3. Synthesis of 3-Alkyl-1,2,4-triazoles[a]



[a]Reagents and conditions: (a) P_2S_5, toluene, 70 °C; (b) MeI, Cs_2CO_3, MeCN, 50 °C; (c) phenoxyacetic hydrazide, DMF, 120 °C.

K. Microwave irradiation was crucial for the outcome of the reaction in order to overcome the poisoning effect of sulfur(II) on palladium catalysts.[25,26] Differently, biphenyl derivatives **91**−**93** were obtained by reaction of the pinacol-boronate **90** with the suitable *p*-bromophenyl derivatives **L** in analogous conditions.

RESULTS AND DISCUSSION

Our initial work focused on exploring variants of the three different arms branching off from the triazole system, namely the thioalkylic chain at position 3, the aromatic ring at position 4, and the phenoxy moiety at the left-hand side, and studying the effect these parts of the molecule have on potency. A first set of analogues was designed in order to understand the importance of the alkylsulfanyl group at position 3. Therefore,

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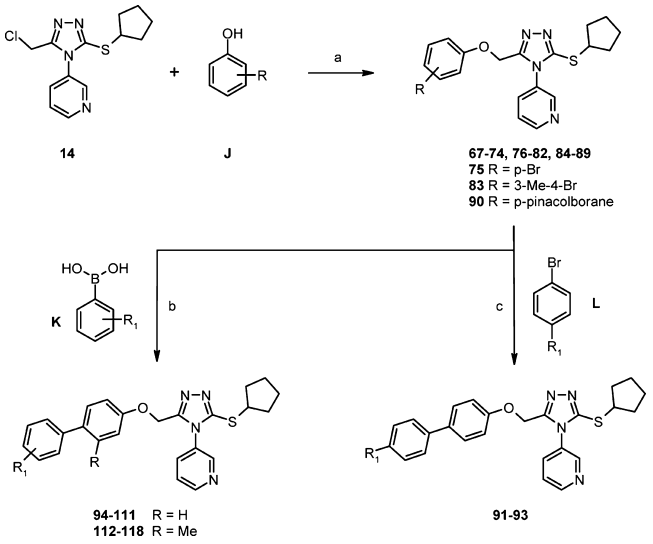
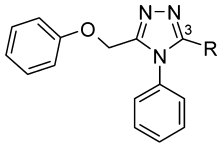
Scheme 4. Variations at the Left-Hand Side[a]



[a]Reagents and conditions: (a) K_2CO_3, DMF, 80−100 °C; (b) from **75**, **83**, Pd(dppf)Cl_2·CH_2Cl_2, Cs_2CO_3, MeCN/H_2O, 100 °C, microwave irradiation; (c) from **90**, Pd(dppf)Cl_2·CH_2Cl_2, Cs_2CO_3, MeCN/H_2O, 100 °C, microwave irradiation.

the isopropyl chain of the original hit was removed or replaced with alkylic residues of different size. As can be seen from Table 1, the presence of a lipophilic group in this position was required. Compounds **18** and **19** that respectively lack the alkyl chain or the whole alkylsulfanyl group were inactive at the highest tested concentration (30 μM). Furthermore, the size of the alkyl chain was found to be critical. Residues smaller than the isopropyl led to less active compounds (**2**, **3** vs **1**), while larger groups (**4**−**7**) improved activity, reaching the optimal size with cyclopentyl, as in compound **6**. In this respect, aliphatic rings directly linked to sulfur represented the best arrangement and were preferred to aliphatic chains (**5** vs **4**) and to rings with one carbon atom spacer (**5**, **7** vs **8**, **9**). Aromatic rings at this position were found to be detrimental (**10** vs **7**), while benzyl (**11**) was probably too large to be accommodated in the hypothetical lipophilic pocket. Sulfur always represents a possible liability from the metabolic point of view. Actually, compound **17**, one of the potential metabolites of **1**, turned out to be inactive. For this reason, we decided to investigate, early on in the optimization stage, sulfur replacement by other isosteric atoms such as oxygen, carbon, and nitrogen. As evident from Table 1, none of the considered isosteric replacements (**20**−**22**, **61**−**65**) gave compounds as active as the parent sulfanyl derivatives. In fact, structure−activity relationship results indicate rather strict structural requirements for this region of the molecule with the cyclopentylsulfanyl group as the optimal substituent among the different arrangements tried at this position.

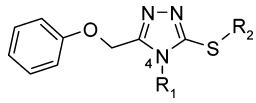
Table 1. SAR:[a] **Variations at Position 3**



ID	R	VCP IC_{50} (μM)	ID	R	VCP IC_{50} (μM)
1	*S*-isopropyl	2.69	**17**	SO_2-isopropyl	>30
2	*S*-methyl	>30	**18**	SH	>30
3	*S*-ethyl	9.92	**19**	H	>30
4	*S*-isobutyl	1.29	**20**	*O*-isopropyl	>30
5	*S*-cyclobutyl	0.96	**21**	*O*-cyclopentyl	2.25
6	*S*-cyclopentyl	0.72	**22**	*O*-cyclohexyl	1.96
7	*S*-cyclohexyl	0.96	**61**	CH_2-cyclopentyl	1.97
8	*S*-CH_2-cyclobutyl	1.15	**62**	CH_2-cyclohexyl	4.30
9	*S*-CH_2-cyclohexyl	>30	**63**	NH-isopropyl	>30
10	*S*-Ph	3.79	**64**	NH-cyclopentyl	2.85
11	*S*-Bn	>30	**65**	NH-cyclohexyl	3.45

[a]Values are the means of two or more experiments.

Table 2. SAR:[a] **Variations at Position 4**



R_2 = isopropyl			R_2 = cyclopentyl		
ID	R_1	VCP IC_{50} (μM)	ID	R_1	VCP IC_{50} (μM)
1	Ph	2.69	**6**	Ph	0.72
23	Ph-*o*-F	19.43	**36**	Ph-*m*-Cl	0.74
24	Ph-*o*-OMe	>30	**37**	Ph-*m*-Br	0.96
25	Ph-*m*-Cl	1.88	**38**	Ph-*m*-Me	2.78
26	Ph-*m*-F	2.06	**39**	Ph-*m*-NO_2	2.81
27	Ph-*m*-Br	2.32	**40**	Ph-*m*-CF_3	>30
28	Ph-*m*-OMe	16.22	**46**	Ph-*m*-NH_2	1.29
29	Ph-*m*-CF_3	>30	**47**	Ph-*m*-N_3	2.40
30	Ph-*p*-F	19.60	**41**	Ph-3,5-Cl	>30
31	Ph-*p*-Cl	>30	**42**	cyclohexyl	>30
32	Ph-*p*-OMe	>30	**43**	2-pyridyl	>30
33	Me	>30	**44**	3-pyridyl	0.47
34	Bn	>30	**45**	CH_2-2-furanyl	>30
35	3-pyridyl	2.69			

[a]Values are the means of two or more experiments.

Next we wanted to investigate the effect of the substituent at position 4 of the triazolic ring (Table 2). This was initially done with a set of compounds (**23**−**35**) bearing the isopropylsulfanyl group of the original hit and then with a second set of compounds (**36**−**47**) possessing the optimized cyclopentylsulfanyl group. The phenyl ring at position 4 emerged to be mandatory for biochemical activity and seems to have an optimal shape to interact with the protein. Indeed, compound **33**, which possesses a methyl instead of phenyl, is inactive as is compound **34**, where the benzyl group is likely too large and not sufficiently flat. This also applies to compounds **42** and **45** bearing cyclohexyl and furanyl-methyl at the same position. Decoration of the phenyl ring with various substituents did not prove to be efficacious in either series. Ortho- and para-substitutions were definitely detrimental, while meta-substituents did not lead to significant improvements in activity. In contrast, 3-pyridyl proved to be a profitable replacement for phenyl. Although the activity was in the same order of magnitude (**35** vs **1**) or slightly better (**44** vs **6**), the use of 3-pyridyl allowed improvement of aqueous solubility which was of borderline acceptability due to the lipophilic character of this

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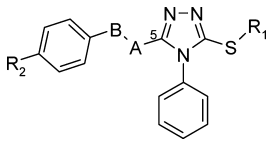
class of compounds. Interestingly, the 2-pyridyl isomer **43**, was inactive.

At the same time, we drew our attention to the left-hand side of the molecule, examining the effect of modifications to the

Table 3. SAR:[a] Effect of the Linker at Position 5

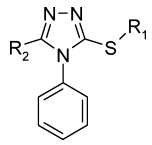


ID	R_1	A–B	R_2	VCP IC_{50} (μM)
1	isopropyl	CH_2O	H	2.69
48	isopropyl	CH_2S	H	7.73
49	isopropyl	CH_2CH_2	H	11.78
50	isopropyl	CH_2OCH_2	H	26.72
51	isopropyl	CH_2	H	>30
52	isopropyl	$CH_2N(Me)$	H	>30
58	isopropyl	CH2NH	H	11.17
53	cyclopentyl	CH_2O	Br	0.44
54	cyclopentyl	CONH	Br	18.64
55	cyclopentyl	CH=CH	Br	2.86

[a]Values are the means of two or more experiments.

oxymethylene linker at position 5 between the phenyl and the central scaffold (Table 3). Also in this case we tried various isosteric replacements, moving from the original oxygen (**1**) to sulfur (**48**), nitrogen (**58**, **52**), and carbon (**49**). In none of these cases did we observe any improvement of activity. A similar outcome was obtained elongating the oxymethylene linker by one carbon atom (**50**) or removing the oxygen (**51**). In addition, the oxymethylene linker was replaced with other spacers that should orientate substituents in the same direction, such as an amidic bond (**54**) and a trans double bond (**55**), without improvement of activity as compared to compound **53**.

Table 4. SAR:[a] Importance of the Aromatic Moiety at the Left-Hand Side



ID	R_1	R_2	VCP IC_{50} (μM)
1	isopropyl	CH_2OPh	2.70
59	isopropyl	CH_2OH	>30
60	isopropyl	CH_2O-cyclohexyl	>30
66	isopropyl	CH_2O-3-pyridyl	5.56
6	cyclopentyl	CH_2OPh	0.72
56	cyclopentyl	2-benzofuranyl	1.94
57	cyclopentyl	2-furanyl	8.72

[a]Values are the means of two or more experiments.

As for the importance of the contiguous phenyl, the set of compounds shown in Table 4 clearly indicate that its removal (**59**) as well as its replacement with a cyclohexyl moiety (**60**) caused a marked drop of activity. Changing phenyl with pyridine did not prove to be effective in this case, and compound **66** was actually 2-fold less active than its phenylic counterpart **1**. Compounds **56** and **57** were synthesized in the attempt to incorporate an oxymethylene linker into a rigid heterocyclic system. Interestingly, these compounds maintained some activity but were less active than the reference compound **6**.

This preliminary expansion allowed us on one hand to identify the basic structural requirements for this class in order to maintain activity and on the other to optimize substituents at position 3 and 4 of the central triazolic scaffold. In this respect, compound **44** represented a good prototype to start further chemical expansion. Compound **44**, with a biochemical IC_{50} of 0.47 μM, was about 5-fold more potent than the original hit **1** but was still devoid of antiproliferative activity (IC_{50} > 20 μM on HCT-116 cell line) despite good Caco-2 cell permeability data (not shown). This clearly indicated the need to further improve potency. To achieve this goal, an extensive expansion of the left-hand part of the molecule was undertaken. This region was particularly amenable to expansion by parallel chemistry through the advanced intermediate **14** that was reacted with a series of commercially available phenols (Scheme 4). More than 200 phenols were selected based on chemical diversity, and results for the most representative compounds are shown in Table 5. Even though a clear structure–activity relationship could not be discerned, ortho-substituents on phenyl, as for compound **67**, were generally detrimental while the overall activity of meta- and para-substituted derivatives (**68**–**79**) was similar or slightly better (with the exception of compound **72**) than the reference compound **44**, especially when lipophilic substituents, such as halogens, were considered. The combination of some of the most interesting groups identified at meta- and para-positions resulted in a small series of derivatives (Table 5, **80**, **82**–**89**) displaying, together with compound **75**, significant antiproliferative activity (<20 μM) against HCT-116 cell lines for the first time in this class. Interestingly, cell activity could be observed only for derivatives with biochemical IC_{50} generally below 300 nM, thus fixing such value as a necessary prerequisite for this class of compounds. To unequivocally associate antiproliferative activity with VCP inhibition, modulation of the most sensitive biomarkers was assessed upon treatment of HCT-116 cells with increasing doses of the representative compound **86**. Previous siRNA experiments (Supporting Information Figure S6, p S26 and Magnaghi et al., manuscript submitted) had clearly shown that VCP ablation induced accumulation of poly ubiquitinated proteins and Cyclin E, as well as activation of the unfolded protein response, as assessed by induction of C/EBP homologous protein transcription factor (CHOP, also known as Gadd153). Western blot analysis of HCT-116 cells treated with compound **86** showed dose-dependent accumulation of poly ubiquitinated proteins and of Cyclin E and clear induction of CHOP at doses consistent with the antiproliferative IC_{50} (Figure 3).

The real breakthrough of this expansion was however represented by compound **81** characterized by a biphenyl system with a cyano group at para-position. This compound, despite a biochemical potency comparable to **86**, possessed significantly improved antiproliferative activity (IC_{50} = 4.07 μM on the HCT-116 cell line). Considering the increased lipophilic character of **81**, this result may be simply explained in terms of improved cell permeability. On the other hand, it is likely that additional factors such as binding to adaptor proteins or post-translational modifications[27] may play a role in determining the real activity in the cellular environment that cannot be appreciated in a cell-free system. Alternatively, one could postulate that the additional phenyl ring might lead to a better

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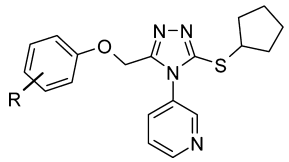
Table 5. SAR:[a] Expansion at the Left-Hand Side



ID	R	VCP IC_{50} (μM)	HCT-116 IC_{50}(μM)	ID	R	VCP IC_{50} (μM)	HCT-116 IC_{50}(μM)
44	H	0.468	>20	**80**	3-F-4-Cl	0.071	14.92
67	*o*-Cl	1.277	>20	**81**	*p*-(Ph-*p*-CN)	0.088	4.07
68	*m*-Cl	0.279	>20	**82**	3,4-Cl	0.090	14.35
69	*m*-Br	0.283	>20	**83**	3-Me-4-Br	0.100	15.69
70	*m*-F	0.379	>20	**84**	3,5-Me-4-Cl	0.134	13.96
71	*m*-Me	0.524	>20	**85**	3-Cl-4-Me	0.150	15.14
72	*m*-CN	3.129	>20	**86**	3-Me-4-Cl	0.184	18.22
73	*m*-OMe	0.375	>20	**87**	3-Br-5-F	0.225	15.36
74	*p*-Cl	0.140	>20	**88**	3,5-Cl	0.257	18.13
75	*p*-Br	0.168	18.09	**89**	3-Me-4-F	0.443	>20
76	*p*-F	0.604	>20				
77	*p*-Me	0.220	>20				
78	*p*-CN	0.609	>20				
79	*p*-OMe	0.587	>20				

[a]Values are the means of two or more experiments.

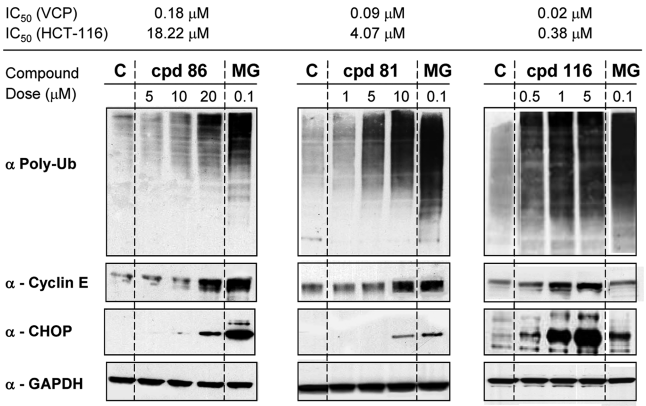


Figure 3. Analysis of biomarkers modulation upon treatment with VCP inhibitors. HCT-116 cells were treated with increasing doses of inhibitors for 8 h. Cells were then rinsed with PBS and lysed. Then 20 μg of lysate proteins were fractionated in SDS PAGE and subjected to immunoblotting with the indicated antibodies. C stands for control; MG stands for the proteasome inhibitor MG-132 used as a standard.

association with the endoplasmic reticulum membrane system where VCP in part resides and to selective interference with the function of membrane-associated VCP, as has been suggested for the aromatic domain of Eeyarestatin.[9]

Indeed, the increased cellular potency of compound **81** was accompanied by a VCP-related mechanism of action because biomarker modulation could in this case be appreciated starting from 5 μM, consistent with the antiproliferative IC_{50} of the compound (Figure 3). To gain better understanding of the role of the *para*-cyano biphenyl arrangement, a small set of analogues (Table 6) was synthesized according to the same procedure reported in Scheme 4. The results obtained showed that the cyano group could conveniently be replaced by other hydrogen-bond acceptor groups, such as amides (**94**, **95**), hydroxamates (**91**), ketones (**92**, **97**), sulfones (**96**), and sulfonamides (**93**), while other chemical functionalities (**98**–**105**) were less effective. The biphenyl system proved to be important for activity, likely acting as a spacer and setting the terminal group at position para at an optimal distance. Notably, compound **106**, without any substituent, was considerably less active than **81**, as well as compounds where the terminal group was shifted to ortho- (**107**, **108** vs **81**, **97**) or meta-position (**109**, **110**, **111** vs **81**, **97**, **96**).

As a next step, by analogy with compounds of Table 5, we considered the opportunity to investigate the effect of additional substituents on the proximal ring of the biphenyl system. Given that substitutions at position ortho to the oxygen (position 2′) did not prove to be effective, we attempted to probe the effect of substitution at position 3′ by introducing a methyl group in some of the most interesting previously identified derivatives (Table 7). This might also provide insight on the effect of disrupting planarity of the biphenyl system.

Interestingly, even though biochemical activity was generally maintained in the same range (**112**–**118** vs **81**, **91**, **93**, **95**–**98**), this introduction resulted in an improvement of the antiproliferative activity for some representatives and was particularly significant for derivative **116** (IC_{50} = 0.38 μM on HCT-116 cell line), which was 1 order of magnitude more active in cells than the parent compound **96**.

For this compound, we also observed a clear dose-dependent modulation of key cellular VCP biomarkers starting from a concentration of 0.5 μM (Figure 3). Compound **116** was also profiled against other representative AAA ATPases (NSF, SPATA5, VPS4B, and cdc6), HSP90, as well as a panel of 50 kinases and did not display any measurable cross-reactivity up to the highest tested concentration of 10 μM (data not shown). Similarly to the original hit **1**, the potency of compound **116** in standard assay conditions was unchanged at saturating ATP concentration (Supporting Information, Table S1, p S26), indicating a mechanism of noncompetitive inhibition for this class. To assess the feasibility for an in vivo efficacy study, pharmacokinetic parameters of compound **116** were evaluated following intravenous (iv) and oral (po) administration in mice (Table 8). After iv dosing, compound **116** was found to be well distributed in tissues (volume of distribution about 10 times the

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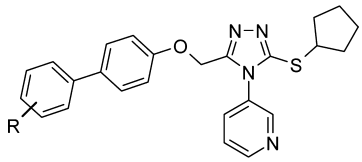
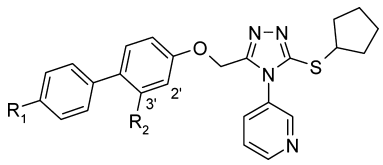
Table 6. SAR:[a] Variations on the Biphenyl Series



ID	R	VCP IC_{50} (μM)	HCT-116 IC_{50}(μM)	ID	R	VCP IC_{50} (μM)	HCT-116 IC_{50}(μM)
81	*p*-CN	0.088	4.07	**101**	*p*-NH_2	0.262	>10
91	*p*-CONHOMe	0.043	3.13	**102**	*p*-Br	0.523	>10
92	*p*-$COCH_2OH$	0.048	1.74	**103**	*p*-F	0.683	>10
93	*p*-SO_2NH_2	0.071	1.37	**104**	*p*-SMe	0.976	>10
94	*p*-$CONH_2$	0.041	5.52	**105**	*p*-CF_3	1.236	>10
95	*p*-CONHMe	0.042	6.44	**106**	H	2.031	>10
96	*p*-SO_2Me	0.063	3.03	**107**	*o*-CN	0.536	>10
97	*p*-COMe	0.065	7.39	**108**	*o*-COMe	0.379	>10
98	*p*-CHO	0.112	9.17	**109**	*m*-CN	1.394	>10
99	*p*-NO_2	0.162	>10	**110**	*m*-COMe	0.451	>10
100	*p*-OMe	0.172	>10	**111**	*m*-SO_2Me	0.908	>10

[a]Values are the means of two or more experiments.

Table 7. SAR:[a] Effect of the *ortho*-Methyl on the Biphenyl System



	R_2 = H			R_2 = Me		
R_1	ID	VCP IC_{50} (μM)	HCT-116 IC_{50}(μM)	ID	VCP IC_{50} (μM)	HCT-116 IC_{50}(μM)
CN	**81**	0.088	4.07	**112**	0.071	2.02
CONHOMe	**91**	0.043	3.13	**113**	0.058	4.60
SO_2NH_2	**93**	0.071	1.37	**114**	0.025	1.82
CONHMe	**95**	0.042	6.44	**115**	0.054	4.98
SO_2Me	**96**	0.063	3.03	**116**	0.024	0.38
COMe	**97**	0.065	7.39	**117**	0.053	1.48
CHO	**98**	0.112	9.17	**118**	0.067	3.16

[a]Values are the means of two or more experiments.

total body water) but with modest systemic exposure, probably due to high clearance (115 mL/min/kg), which was consistent with the high in vitro intrinsic clearance observed during stability testing in the presence of human liver microsomes (Cl_{int} = 400 mL/min/kg). Following oral administration, compound **116** showed modest bioavailability (F = 16.4%), possibly due to extensive first-pass metabolism and/or solubility issues (about 7 μM in aqueous ammonium acetate buffer at pH 7). On the other hand, cell permeability, measured in Caco-2 cells, was good (P_{app} = 21.6 cm 10^{-6}/s).

Further work to improve PK and ADME characteristics of compound **116**, particularly with respect to solubility and metabolic stability issues, will be undertaken in order to identify representatives with a PK profile more appropriate for in vivo efficacy studies.

Compound **116** is the most potent VCP inhibitor disclosed to date and thus represents a valuable tool for investigating the biological consequences of inhibiting this enzyme in cells and gather further insight into the rationale for VCP-targeted cancer therapy.

CONCLUSIONS

In this report, the first known allosteric inhibitors of VCP ATPase activity are described. Starting from the basic framework of alkylsulfanyltriazole **1**, which emerged from HTS as a marginally active hit and which was devoid of antiproliferative activity, the structure−activity relationships associated with this class of compounds were investigated. This allowed determination of the major chemical features within the class that are responsible for inhibition of VCP ATPase activity, particularly:

- the cycloalkylic group, preferably cyclopentyl, directly linked to sulfur at position 3 of the scaffold
- the 3-pyridyl group at position 4, able to confer potency and solubility
- the aromatic ring, preferably phenyl, linked to the oxymethylene linker at position 5 of the triazole

Further parallel expansion and optimization of the left-hand part of the molecule following a traditional iterative medicinal chemistry approach led to the identification of low nanomolar inhibitors of VCP ATPase activity. Compound **116**, the best representative of this class to date, inhibits VCP ATPase with

Table 8. Pharmacokinetic Parameters of Compound 116 Following iv Bolus Single Dose and Oral Single Dose in Harlan nu/nu Mice[a]

PK data (iv), dose:[b] 1 mg/kg					PK data (per os), dose:[c] 10 mg/kg				
C_{max} (μM)	AUC_∞ (μM h)	CL (mL/min/kg)	Vss (mL/kg)	$t_{1/2}$ (h)	C_{max} (μM)	t_{max} (h)	AUC_∞ (μM h)	$t_{1/2}$ (h)	F[d] (%)
0.30 ± 0.08	2.26 ± 0.07	115 ± 31	5882 ± 1298	0.9	0.06 ± 0.02	0.8	0.43 ± 0.11	6.0	16.4

[a]n = 3 animals per study. [b]Dosed in 10% Tween 80 in 5% dextrose. [c]Dosed in 0.5% Methocel. [d]Bioavailability.

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an IC_{50} of 24 nM and possesses antiproliferative activity in the submicromolar range (IC_{50} = 0.38 μM on HCT-116 cell lines), which is associated, at consistent concentrations, with modulation of known VCP biomarkers.

Further work to optimize ADME and PK characteristics of this class will next be undertaken for identification of candidates suitable for in vivo efficacy studies and will be described in due course.

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. When necessary, compounds were purified by preparative HPLC on a Waters Symmetry C18 (19 mm × 50 mm, 5 μm) column using a Waters preparative HPLC 600 equipped with a 996 Waters PDA detector and a Micromass model A ZMD single quadrupole mass spectrometer, with electrospray ionization, in the positive mode, was used. Mobile phase A was water and 0.01% trifluoroacetic acid and mobile phase B was acetonitrile. The following conditions were used: a gradient from 10 to 90% B in 8 min and held at 90% B for 2 min; a flow rate of 20 mL/min. Column chromatography was conducted either under medium pressure on silica (Merck silica gel 40−63 μm) or on prepacked silica gel cartridges (Biotage) or on a Horizon system. ^1H and ^{13}C NMR spectra were recorded on a Varian Inova 400 spectrometer operating respectively at 400.5 MHz for proton and 100.7 MHz for carbon and equipped with a 5 mm $^1H\{^{15}N-^{31}P\}$ z-axis- PFG indirect detection probe. Residual solvent signal was used as reference (DMSO-d_6 at 2.50 ppm for ^1H and 39.5 ppm for ^{13}C). Data are reported as follows: chemical shift δ (in ppm), multiplicity (s = singlet, d = doublet, t = triplet, bs = broad singlet, m = multiplet), coupling constants J (in Hz), and number of protons. Low-resolution mass spectral (MS) data were determined on a Finnigan MAT LCQ ion trap instrument equipped with ESI. High-resolution mass spectra (HRMS) were obtained on a Waters Q-TOF Ultima instrument equipped with ESI and using reserpine (MW 609.28065) for lock mass correction. Thin-layer chromatography was performed on Merck silica gel 60 plates coated with a 250 μM layer with a fluorescent indicator. Components were visualized by UV light (λ = 254 and 366 nm) and iodine vapors.

The following abbreviations for solvents and reagents are used: EtOH = ethanol, DCM = dichloromethane, EtOAc = ethyl acetate, THF = tetrahydrofuran, DMF = dimethylformamide, Et_2O = diethyl ether, MeOH = methanol, MeCN = acetonitrile, TMOF = triethyl orthoformate, Pd(dppf)Cl_2·DCM = [1,1′-bis(diphenylphosphino)-ferrocene]dichloro palladium(II) complex with dichloromethane, DIPEA = *N*-ethyldiisopropylamine.

5-Benzyloxymethyl-4-pyridin-3-yl-4H-[1,2,4]triazole-3-thiol (General Formula C, Scheme 1). A solution of benzyloxyacetic acid (5 g, 30 mmol) and *p*-toluenesulfonic acid monohydrate in EtOH (25 mL) was heated at reflux for 3 h. The solvent was evaporated under vacuum, the residue dissolved in DCM (30 mL) and washed with satd aq Na_2CO_3, water, and brine. The organic phase was dried with anhydrous sodium sulfate and evaporated under vacuum. Benzyloxyacetic acid ethyl ester (4.87 g, 84% yield) was obtained as colorless oil. The ester (4.87 g, 25.09 mmol) was dissolved in EtOH (20 mL) and hydrazine monohydrate added (2.44 mL, 50.18 mmol). The mixture was heated at reflux for 3 h, then the solvent was evaporated under vacuum. Water (50 mL) was added and the reaction was extracted with DCM (5 × 20 mL). The organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated under vacuum. Benzyloxyacetic acid hydrazide (4.24 g, 94% yield) was obtained as white solid. A mixture of benzyloxyacetic acid hydrazide (3.56 g, 19.78 mmol) and 3-pyridyl isothiocyanate (2.69 g, 19.78 mmol) in EtOH (40 mL), was heated at reflux for 1 h, then cooled at room temperature. The suspension was filtered, and the solid was washed with EtOH and dried. 2-[(Benzyloxy)acetyl]-*N*-(pyridin-3-yl)-hydrazinecarbothioamide (5.82 g, 85% yield) was obtained as white solid. Then 1 N NaOH (30 mL) was added to this solid and the mixture was heated at reflux for 1 h. The resulting solution was cooled at room temperature and acidified to pH 6 with 1 M HCl. The precipitate was filtered, washed with water, and dried to obtain the title compound (4.06 g, 74% yield) as white solid. ^1H NMR (400 MHz), δ (ppm, DMSO-d_6): 14.06 (bs, 1H), 8.72 (m, 1H), 8.65 (d, J = 2.32 Hz, 1H), 7.94 (m, 1H), 7.61 (m, 1H), 7.30−7.25 (m, 3H), 7.08 (m, 2H), 4.40 (s, 2H), 4.35 (s, 2H). MS (ESI) m/z 299 [(M + H)$^+$].

By analogous procedure, starting from the suitable carboxylic acid and the appropriate isothiocyanate, all the compounds of general formula C, Scheme 1 (see Supporting Information, p S2) and compound **18** were prepared.

*5-Phenoxymethyl-4-phenyl-4H-[1,2,4]triazole-3-thiol (**18**).* Yield 2 g, 70%. ^1H NMR (400 MHz), δ (ppm, DMSO-d_6): 14.03 (bs, 1H), 7.55−7.47 (m, 3H), 7.43 (m, 2H), 7.23 (m, 2H), 6.94 (m, 1H), 6.82 (m, 2H), 4.96 (s, 2H). MS (ESI) m/z 284 [(M + H)$^+$].

*3-Isopropylsulfanyl-5-phenoxymethyl-4-phenyl-4H-[1,2,4]-triazole (**1**).* To a solution of 5-phenoxymethyl-4-phenyl-4*H*-[1,2,4]-triazole-3-thiol (**18**) (0.10 g, 0.35 mmol) in anhydrous DMF (3 mL), 2-bromopropane (0.07 mL, 0.7 mmol) and potassium carbonate (0.12 g, 0.88 mmol) were added. The mixture was stirred at room temperature for 15 h, then the reaction was poured in water and extracted with EtOAc (2 × 10 mL). The organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated. The crude residue was purified by silica gel column chromatography (DCM/MeOH 97:3) to afford the title compound (0.08 g, 70% yield) as white solid. ^1H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.57−7.52 (m, 3H), 7.46−7.43 (m, 2H), 7.23 (m, 2H), 6.93 (m, 1H), 6.85 (m, 2H), 5.07 (s, 2H), 3.70 (m, 1H), 1.31 (d, J = 6.71 Hz, 6H). MS (ESI) m/z 326 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{18}H_{20}N_3OS^+$ [(M + H)$^+$] 326.1322, found 326.1321.

By analogous procedure, starting from the suitable 2,4-dihydro-[1,2,4]triazole-3-thione (general formula C, Scheme 1) and the appropriate alkyl bromide or alkyl iodide, derivatives **2**−**5**, **7**−**9**, **11**, **23**−**43**, **45**, **48**−**51**, **53**−**57** (see Supporting Information, p S6), and the following compounds were prepared:

*3-Cyclopentylsulfanyl-5-phenoxymethyl-4-phenyl-4H-[1,2,4]-triazole (**6**).* Yield 0.12 g, 70%. ^1H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.58−7.52 (m, 3H), 7.47−7.43 (m, 2H), 7.23 (m, 2H), 6.93 (m, 1H), 6.85 (m, 2H), 5.06 (s, 2H), 3.84 (m, 1H), 2.07 (m, 2H), 1.65−1.53 (m, 6H). ^{13}C NMR (100.7 MHz), δ (ppm, DMSO-d_6): 157.3, 151.8, 151.4, 132.8, 129.8, 129.6, 129.3, 127.1, 121.4, 114.8, 59.8, 45.5, 33.1, 24.1. MS (ESI) m/z 352 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{20}H_{22}N_3OS^+$ [(M + H)$^+$] 352.1478; found 352.1494. Melting point 129−130 °C.

*3-(3-Benzyloxymethyl-5-cyclopentylsulfanyl-[1,2,4]triazol-4-yl)-pyridine (**12**).* Yield 3.36 g, 67%. ^1H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.77 (m, 1H), 7.96 (m, 1H), 7.61 (m, 1H), 7.31−7.25 (m, 3H), 7.08 (m, 2H), 4.53 (s, 2H), 4.36 (s, 2H), 3.80 (m, 1H), 2.04 (m, 2H), 1.65−1.49 (m, 6H). MS (ESI) m/z 367 [(M + H)$^+$].

*3-(3-Cyclopentylsulfanyl-5-phenoxymethyl-[1,2,4]triazol-4-yl)-pyridine (**44**).* Yield 0.05 g, 50%. ^1H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.72−8.68 (m, 2H), 7.99 (m, 1H), 7.61 (m, 1H), 7.23 (m, 2H), 6.93 (m, 1H), 6.84 (m, 2H), 5.13 (s, 2H), 3.82 (m, 1H), 2.07 (m, 2H), 1.68−1.53 (m, 6H). ^{13}C NMR (100.7 MHz), δ (ppm, DMSO-d_6): 157.1, 151.9, 151.7, 150.8, 147.7, 135.2, 130.0, 129.4, 124.3, 121.4, 114.7, 59.8, 46.0, 33.1, 24.0. MS (ESI) m/z 353 [(M + H)$^+$]. HRMS

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(ESI) calculated for $C_{19}H_{21}N_4OS^+$ [(M + H)$^+$] 353.1431; found 353.1435. Melting point 83−84 °C.

*3-Phenoxymethyl-4-phenyl-4H-[1,2,4]triazole (**19**).* To a solution of **18** (0.6 g, 2.12 mmol) in DCM (30 mL), cooled at 0−5 °C, a solution of 30% aq H_2O_2 (0.6 mL, 5.3 mmol) in acetic acid (3.5 mL) was added dropwise. The reaction was stirred at room temperature for 1 h, then was washed with 1 N NaOH (50 mL). The organic phase was dried with anhydrous sodium sulfate and evaporated under vacuum. The title compound (0.51 g, 95% yield) was obtained as yellowish solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.87 (s, 1H), 7.58−7.51 (m, 5H), 7.26 (m, 2H), 6.92 (m, 1H), 5.22 (s, 2H). MS (ESI) m/z 252 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{15}H_{14}N_3O^+$ [(M + H)$^+$] 252.1132; found 252.1124

*3-Methanesulfonyl-5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazole (**16**).* To a solution of **2** (0.48 g, 1.61 mmol) in DCM (10 mL), 3-chloroperbenzoic acid (1.11 g, 6.44 mmol) was added. The reaction was stirred at room temperature for 15 h, then diluted with DCM (40 mL) and washed with 10% aq sodium thiosulfate (20 mL), satd aq $NaHCO_3$ (20 mL), water, and brine. The organic phase was dried with anhydrous sodium sulfate and evaporated under vacuum. The crude residue was ground with Et_2O (10 mL) to obtain the title compound (0.37 g, 70% yield) as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.60−7.53 (m, 5H), 7.24 (m, 2H), 6.94 (m, 1H), 6.87 (m, 2H), 5.12 (s, 2H), 3.46 (s, 3H). MS (ESI) m/z 330 [(M + H)$^+$].

By analogous procedure, starting from **1** the following compound was obtained:

*3-Phenoxymethyl-4-phenyl-5-(propane-2-sulfonyl)-4H-[1,2,4]-triazole (**17**).* Yield 0.11 g, 68%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.55−7.48 (m, 5H), 7.21 (m, 2H), 6.91 (m, 1H), 6.83 (m, 2H), 5.07 (s, 2H), 3.54 (m, 1H), 1.23 (d, J = 6.84 Hz, 6H). MS (ESI) m/z 358 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{18}H_{20}N_3O_3S^+$ [(M + H)$^+$] 358.1220; found 358.1228.

*3-Isopropoxy-5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazole (**20**).* To a solution of 2-propanol (0.07 mL, 0.91 mmol) in anhydrous THF (2 mL), 60% sodium hydride (0.03 g, 0.76 mmol) was added. The mixture was stirred at room temperature for 15 min, then **16** (0.1 g, 0.30 mmol) was added in portions. The reaction was stirred at room temperature for 5 h, then poured in water (20 mL) and extracted with EtOAc (2 × 10 mL). The organic phase was dried with anhydrous sodium sulfate and evaporated under vacuum. The crude residue was purified by silica gel column chromatography (EtOAc/exane 7:3) to afford the title compound (0.04 g, 42% yield) as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.54−7.44 (m, 5H), 7.24 (m, 2H), 6.93 (m, 1H), 6.88 (m, 2H), 5.09 (m, 1H), 4.99 (s, 2H), 1.32 (d, J = 6.10 Hz, 6H). MS (ESI) m/z 310 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{18}H_{20}N_3O_2^+$ [(M + H)$^+$] 310.1550; found 310.1548.

By analogous procedure, starting from **16** and the suitable alcohols or thiophenol the following compounds were obtained:

*3-Cyclopentyloxy-5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazole (**21**).* Yield 0.07 g, 69%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.54−7.42 (m, 5H), 7.24 (m, 2H), 6.93 (m, 1H), 6.88 (m, 2H), 5.29 (m, 1H), 4.99 (s, 2H), 1.91 (m, 2H), 1.77 (m, 2H), 1.56 (m, 4H). MS (ESI) m/z 336 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{20}H_{22}N_3O_2^+$ [(M + H)$^+$] 336.1707; found 336.1704.

*3-Cyclohexyloxy-5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazole (**22**).* Yield 0.04 g, 38%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.54−7.44 (m, 5H), 7.24 (m, 2H), 6.93 (m, 1H), 6.88 (m, 2H), 4.99 (s, 2H), 4.86 (m, 1H), 1.95 (m, 2H), 1.65−1.20 (m, 8H). MS (ESI) m/z 350 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{21}H_{24}N_3O_2^+$ [(M + H)$^+$] 350.1863; found 350.1862.

*3-Phenoxymethyl-4-phenyl-5-phenylsulfanyl-4H-[1,2,4]triazole (**10**).* Yield 0.08 g, 72%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.51−7.44 (m, 3H), 7.37−7.28 (m, 5H), 7.25−7.17 (m, 4H), 6.93 (m, 1H), 6.84 (m, 2H), 5.10 (s, 2H). MS (ESI) m/z 360 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{21}H_{18}N_3OS^+$ [(M + H)$^+$] 360.1165; found 360.1168.

*3-(3-Cyclopentylsulfanyl-5-phenoxymethyl-[1,2,4]triazol-4-yl)-phenylamine (**46**).* To a solution of **39** (0.25 g, 0.63 mmol) in a mixture of MeOH (10 mL) and water (3.5 mL), ammonium chloride (0.17 g, 3.16 mmol), and iron powder (0.11 g, 1.89 mmol) were added. The reaction was heated at reflux for 4 h, then cooled at room temperature and filtered on Celite washing with MeOH. The solvent was evaporated under vacuum, and the residue was dissolved in EtOAc (20 mL) and washed with satd aq $NaHCO_3$ (20 mL), water, and brine. The organic phase was dried with anhydrous sodium sulfate and evaporated. The title compound (0.18 g, 78% yield) was obtained as yellowish solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.25 (m, 2H), 7.13 (m, 1H), 6.96−6.90 (m, 3H), 6.67 (m, 1H), 6.51 (m, 1H), 6.47 (m, 1H), 5.48 (bs, 2H), 5.01 (s, 2H), 3.87 (m, 1H), 2.09 (m, 2H), 1.67−1.50 (m, 6H). MS (ESI) m/z 367 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{20}H_{23}N_4OS^+$ [(M + H)$^+$] 367.1587; found 367.1596.

*4-(3-Azido-phenyl)-3-cyclopentylsulfanyl-5-phenoxymethyl-4H-[1,2,4]triazole (**47**).* To a solution of **46** (0.17 g, 0.48 mmol) in 20% aq HCl (5 mL), cooled at 0−5 °C, a solution of sodium nitrite (0.03 g, 0.48 mmol) in water (1 mL) was added dropwise while maintaining the temperature below 5 °C. The reaction was stirred at 0−5 °C for 30 min, then a solution of sodium azide (0.03 g, 0.53 mmol) in water (1 mL) was added dropwise while maintaining the temperature below 5 °C. After 15 min, the mixture was diluted with EtOAc (20 mL) and washed with water (20 mL) and brine. The organic phase was dried with anhydrous sodium sulfate and evaporated under vacuum. The title compound (0.14 g, 74% yield) was obtained as yellowish solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.57 (m, 1H), 7.30−7.22 (m, 5H), 6.94 (m, 1H), 6.87 (m, 2H), 5.10 (s, 2H), 3.84 (m, 1H), 2.07 (m, 2H), 1.67−1.52 (m, 6H). MS (ESI) m/z 393 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{20}H_{21}N_6OS^+$ [(M + H)$^+$] 393.1492; found 393.1482.

*(5-Cyclopentylsulfanyl-4-pyridin-3-yl-4H-[1,2,4]triazol-3-yl)-methanol (**13**).* To a solution of **12** (7.41 g, 20 mmol) in anhydrous DCM (50 mL), cooled at 0−5 °C, 1 M boron trichloride solution in DCM (80 mL, 80 mmol) was added dropwise. The reaction was stirred at 0−5 °C for 2 h, then 33% aq NH_4OH (100 mL) was added dropwise and the mixture was vigorously stirred for 1 h. The organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated under vacuum. The crude residue was purified by silica gel column chromatography (DCM/MeOH 95:5) to afford the title compound (4.21 g, 76% yield) as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.75 (m, 1H), 8.67 (m, 1H), 7.97 (m, 1H), 7.64 (m, 1H), 5.43 (t, J = 5.61 Hz, 1H), 4.45 (d, J = 5.61 Hz, 2H), 3.77 (m, 1H), 2.03 (m, 2H), 1.65−1.51 (m, 6H). MS (ESI) m/z 277 [(M + H)$^+$].

By analogous procedure, starting from **50** the following compound was prepared:

*(5-Isopropylsulfanyl-4-phenyl-4H-[1,2,4]triazol-3-yl)-methanol (**59**).* Yield 0.24 g, 77%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.60−7.53 (m, 3H), 7.43−7.39 (m, 2H), 5.38 (bs, 1H), 4.39 (s, 2H), 3.64 (m, 1H), 1.29 (d, J = 6.71 Hz, 6H). MS (ESI) m/z 250 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{12}H_{16}N_3OS^+$ [(M + H)$^+$] 250.1009; found 250.1005.

*3-(3-Chloromethyl-5-cyclopentylsulfanyl-[1,2,4]triazol-4-yl)-pyridine (**14**).* To a solution of **13** (0.50 g, 1.81 mmol) in anhydrous DCM (40 mL), cooled at 0−5 °C, thionyl chloride (0.20 mL, 2.72 mmol) was added. The reaction was stirred at 0−5 °C for 2 h, then was washed with satd aq $NaHCO_3$, water, and brine. The organic phase was dried with anhydrous sodium sulfate and evaporated under vacuum. The title compound (0.52 g, 98% yield) was obtained as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.79 (m, 1H), 8.73 (m, 1H), 8.03 (m, 1H), 7.67 (m, 1H), 4.80 (s, 2H), 3.82 (m, 1H), 2.05 (m, 2H), 1.65−1.53 (m, 6H). MS (ESI) m/z 295 [(M + H)$^+$].

By analogous procedure, starting from **59** the following compound was prepared:

*3-Chloromethyl-5-isopropylsulfanyl-4-phenyl-4H-[1,2,4]triazole (**15**).* Yield 0.08 g, 75%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.59−7.55 (m, 3H), 7.45−7.38 (m, 2H), 4.72 (s, 2H), 3.75 (m, 1H), 1.32 (d, J = 6.70 Hz, 6H). MS (ESI) m/z 268 [(M + H)$^+$].

*(5-Isopropylsulfanyl-4-phenyl-4H-[1,2,4]triazol-3-ylmethyl)-phenyl-amine (**58**).* To a solution of **15** (0.08 g, 0.28 mmol) in anhydrous DMF (2 mL), aniline (0.05 mL, 0.56 mmol) and potassium carbonate (0.08 g, 0,56 mmol) were added. The reaction was stirred at 80 °C for

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1 h, then was cooled at room temperature, poured in water (20 mL), and extracted with EtOAc (2 × 20 mL). The organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated under vacuum. The crude residue was purified by silica gel column chromatography (DCM/MeOH 97:3) to afford the title compound (0.07 g, 70% yield) as yellowish oil. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.57−7.536 (m, 3H), 7.43−7.39 (m, 2H), 7.02 (m, 2H), 6.55−6.50 (m, 3H), 5.84 (t, J = 5.74 Hz, 1H), 4.20 (d, J = 5.74 Hz, 2H), 3.62 (m, 1H), 1.28 (d, J = 6.84 Hz, 6H). MS (ESI) m/z 325 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{18}H_{21}N_4S^+$ [(M + H)$^+$] 325.1482; found 325.1472.

By analogous procedure, starting from **15** and *N*-methylaniline, the following compound was prepared:

*(5-Isopropylsulfanyl-4-phenyl-4H-[1,2,4]triazol-3-ylmethyl)-methyl-phenyl-amine (**52**).* Yield 0.06 g, 45%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.58−7.53 (m, 3H), 7.37−7.34 (m, 2H), 7.07 (m, 2H), 6.61 (m, 1H), 6.56 (m, 2H), 4.51 (s, 2H), 3.62 (m, 1H), 2.57 (s, 3H), 1.28 (d, J = 6.84 Hz, 6H). MS (ESI) m/z 339 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{19}H_{23}N_4S^+$ [(M + H)$^+$] 339.1570; found 339.1572.

*3-Cyclohexyloxymethyl-5-isopropylsulfanyl-4-phenyl-4H-[1,2,4]-triazole (**60**).* To a solution of cyclohexanol (0.09 mL, 0.89 mmol) in anhydrous THF (2 mL), cooled at 0−5 °C, 60% sodium hydride (0.05 g, 1.34 mmol) was added. The mixture was stirred at 0−5 °C for 15 min, then **15** (0.12 g, 0.45 mmol) was added in portions. The reaction was stirred at reflux for 1 h, then poured in water (20 mL) and extracted with EtOAc (2 × 10 mL). The organic phase was dried with anhydrous sodium sulfate and evaporated under vacuum. The crude residue was purified by silica gel column chromatography (DCM/MeOH 97:3) to afford the title compound (0.10 g, 68% yield) as colorless oil. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.60−7.54 (m, 3H), 7.44−7.39 (m, 2H), 4.43 (s, 2H), 3.66 (m, 1H), 3.09 (m, 1H), 1.68−1.06 (m, 16H). MS (ESI) m/z 332 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{18}H_{26}N_3OS^+$ [(M + H)$^+$] 332.1791; found 332.1797.

By analogous procedure, starting from **15** and 3-hydroxypyridine, the following compound was prepared:

*3-(5-Isopropylsulfanyl-4-phenyl-4H-[1,2,4]triazol-3-ylmethoxy)-pyridine (**66**).* Yield 0.03 g, 31%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.16−8.14 (m, 2H), 7.58−7.52 (m, 3H), 7.47−7.45 (m, 2H), 7.34 (m, 1H), 7.28 (m, 1H), 5.02 (s, 2H), 3.85 (m, 1H), 1.45 (d, J = 6.71 Hz, 6H). MS (ESI) m/z 327 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{17}H_{19}N_4OS^+$ [(M + H)$^+$] 327.4211; found 327.4215.

1-Benzyl-1-isopropyl-3-phenyl-thiourea (General Formula D, Scheme 2). A solution of phenyl isothiocyanate (0.89 mL, 7.40 mmol) and benzylisopropyl amine (1.24 mL, 7.40 mmol) in EtOH (10 mL) was stirred at room temperature for 2 h. The solid precipitated was filtered and washed with EtOH. After drying under vacuum, the title compound (1.42 g, 68% yield) was obtained as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.93 (bs, 1H), 7.34 (m, 2H), 7.26−7.16 (m, 7H), 7.09 (m, 1H), 5.50 (m, 1H), 4.97 (s, 2H), 1.14 (d, J = 6.71 Hz, 6H). MS (ESI) m/z 285 [(M + H)$^+$].

By analogous procedure, starting from phenyl isothiocyanate and the appropriate amine, the following compounds were prepared:

1-Benzyl-1-cyclopentyl-3-phenyl-thiourea (General Formula D, Scheme 2). Yield 1.20 g, 71%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 9.98 (bs, 1H), 7.34 (m, 2H), 7.29−7.16 (m, 7H), 7.10 (m, 1H), 5.45 (m, 1H), 4.99 (s, 2H), 1.85 (m, 2H), 1.63−1.44 (m, 6H). MS (ESI) m/z 311 [(M + H)$^+$].

1-Benzyl-1-cyclohexyl-3-phenyl-thiourea (General Formula D, Scheme 2). Yield 1.10 g, 77%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.96 (bs, 1H), 7.33 (m, 2H), 7.28−7.16 (m, 7H), 7.10 (m, 1H), 5.11 (m, 1H), 5.01 (s, 2H), 1.74−1.68 (m, 3H), 1.58 (m, 2H), 1.43−1.26 (m, 3H), 1.07 (m, 2H). MS (ESI) m/z 325 [(M + H)$^+$].

1-Benzyl-1-isopropyl-2-methyl-3-phenyl-isothiourea (General Formula E, Scheme 2). To a solution of 1-benzyl-1-isopropyl-3-phenyl-thiourea (1.42 g, 5.00 mmol) in anhydrous THF (20 mL), cooled at 0−5 °C, potassium *tert*-butoxide (0.67 g, 6.00 mmol) was added in portions. After a few minutes, methyl iodide (0.37 mL, 6.00 mmol) was added and the reaction was stirred at 0−5 °C for 1 h. The mixture was poured in water (100 mL) and extracted with EtOAc (2 × 2o mL), then the organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated under vacuum. The title compound (1.30 g, 87% yield) was obtained as yellowish oil. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.34−7.27 (m, 4H), 7.22−7.15 (m, 3H), 6.87 (m, 1H), 6.64 (m, 2H), 4.63 (m, 1H), 4.58 (s, 2H), 1.95 (s, 3H), 1.20 (d, J = 6.71 Hz, 6H). MS (ESI) m/z 299 [(M + H)$^+$].

By analogous procedure, the following compounds were prepared:

1-Benzyl-1-cyclopentyl-2-methyl-3-phenyl-isothiourea (General Formula E, Scheme 2). Yield 0.90 g, 80%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.33 (m, 2H), 7.25 (m, 2H), 7.21−7.15 (m, 3H), 6.87 (m, 1H), 6.68 (m, 2H), 4.69 (m, 1H), 4.64 (s, 2H), 1.93 (s, 3H), 1.84 (m, 2H), 1.65−1.48 (m, 6H). MS (ESI) m/z 325 [(M + H)$^+$].

1-Benzyl-1-cyclohexyl-2-methyl-3-phenyl-isothiourea (General Formula E, Scheme 2). Yield 0.75 g, 88%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.32−7.25 (m, 4H), 7.21−7.14 (m, 3H), 6.86 (m, 1H), 6.65 (m, 2H), 4.64 (s, 2H), 4.22 (m, 1H), 1.92 (s, 3H), 1.77−1.70 (m, 4H), 1.57−1.49 (m, 3H), 1.35−1.22(m, 2H), 1.07 (m, 1H). MS (ESI) m/z 339 [(M + H)$^+$].

Benzyl-isopropyl-(5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazol-3-yl)-amine (General Formula F, Scheme 2). To a solution of 1-benzyl-1-isopropyl-2-methyl-3-phenyl-isothiourea (1.29 g, 4.33 mmol) and phenoxy-acetic acid hydrazide (0.72 g, 4.33 mmol) in THF (20 mL), trifluoroacetic acid (0.17 mL, 2.17 mmol) was added. The mixture was heated at reflux for 48 h, then cooled to room temperature and poured in satd aq $NaHCO_3$. The reaction was extracted with EtOAc (2 × 20 mL), the organic phase was washed with brine and dried with anhydrous sodium sulfate. After evaporation under vacuum, the crude residue was purified by silica gel column chromatography (DCM/MeOH 97:3) to afford the title compound (0.97 g, 56% yield) as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.54−7.47 (m, 3H), 7.33−7.25 (m, 5H), 7.23−7.19 (m, 4H), 6.91 (m, 1H), 6.80 (m, 2H), 4.87 (s, 2H), 4.23 (s, 2H), 3.21 (m, 1H), 0.97 (d, J = 6.71 Hz, 6H). MS (ESI) m/z 399 [(M + H)$^+$].

By analogous procedure, starting from the appropriate substituted 2-methyl-3-phenyl-isothiourea, the following compounds were prepared:

Benzyl-cyclopentyl-(5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazol-3-yl)-amine (General Formula F, Scheme 2). Yield 0.75 g, 61%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.51−7.46 (m, 3H), 7.29 (m, 2H), 7.25−7.19 (m, 5H), 7.09 (m, 2H), 6.91 (m, 1H), 6.83 (m, 2H), 4.90 (s, 2H), 4.13 (s, 2H), 3.52 (m, 1H), 1.60−1.53 (m, 6H), 1.35−1.30 (m, 2H). MS (ESI) m/z 425 [(M + H)$^+$].

Benzyl-cyclohexyl-(5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazol-3-yl)-amine (General Formula F, Scheme 2). Yield 0.62 g, 51%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.55−7.46 (m, 3H), 7.34 (m, 2H), 7.27−7.18 (m, 5H), 6.91 (m, 1H), 6.79 (m, 2H), 4.87 (s, 2H), 4.28 (s, 2H), 2.80 (m, 1H), 1.58 (m, 2H), 1.45−1.24 (m, 3H), 1.00−0.80 (m, 5H). MS (ESI) m/z 439 [(M + H)$^+$].

*Isopropyl-(5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazol-3-yl)-amine (**63**).* To a solution of benzyl-isopropyl-(5-phenoxymethyl-4-phenyl-4*H*-[1,2,4]triazol-3-yl)-amine (0.20 g, 0.50 mmol) in formic acid (2 mL), 5% palladium on carbon (50% w/w, 0.10 g) was added. The mixture was heated at 75 °C for 6 h, then cooled at room temperature. The reaction was diluted with MeOH (10 mL), filtered on Celite, and evaporated under vacuum. The crude residue was purified by silica gel column chromatography (DCM/MeOH 96:4) to afford the title compound (0.07 g, 46% yield) as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.52−7.43 (m, 3H), 7.38−7.35 (m, 2H), 7.19 (m, 2H), 6.88 (m, 1H), 6.85 (m, 2H), 5.38 (d, J = 7.81 Hz, 1H), 4.83 (s, 2H), 3.77 (m, 1H), 1.11 (d, J = 6.47 Hz, 6H). MS (ESI) m/z 309 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{18}H_{21}N_4O^+$ [(M + H)$^+$] 309.1710; found 309.1715.

By analogous procedure, the following compounds were prepared:

*Cyclopentyl-(5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazol-3-yl)-amine (**64**).* Yield 0.05 g, 39%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.54−7.45 (m, 3H), 7.41−7.39 (m, 2H), 7.22 (m, 2H), 6.91 (m, 1H), 6.85 (m, 2H), 5.61 (bs, 1H), 4.86 (s, 2H), 3.95 (m, 1H), 1.89 (m, 2H), 1.60 (m, 2H), 1.50−1.43 (m, 4H). MS (ESI) m/z 335 [(M +

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$H)^+$]. HRMS (ESI) calculated for $C_{20}H_{23}N_4O^+$ [$(M + H)^+$] 335.1867; found 335.1862.

*Cyclohexyl-(5-phenoxymethyl-4-phenyl-4H-[1,2,4]triazol-3-yl)-amine (**65**).* Yield 0.06 g, 41%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.54−7.45 (m, 3H), 7.40−7.38 (m, 2H), 7.22 (m, 2H), 6.91 (m, 1H), 6.85 (m, 2H), 5.40 (d, J = 7.69 Hz, 1H), 4.86 (s, 2H), 3.44 (m, 1H), 1.92 (m, 2H), 1.86−1.46 (m, 3H), 1.29−0.99 (m, 5H). MS (ESI) m/z 349 [$(M + H)^+$]. HRMS (ESI) calculated for $C_{21}H_{25}N_4O^+$ [$(M + H)^+$] 349.2023; found 349.2019.

2-Cyclopentyl-N-phenyl-acetamide (General Formula G, Scheme 3). A solution of cyclopentylacetic acid (3.30 mL, 26.31 mmol) in thionyl chloride (10 mL) was heated at reflux for 2 h, then concentrated under vacuum and dried. The acyl chloride, as obtained, was dissolved in anhydrous THF (10 mL) and added dropwise to a solution of aniline (2 mL, 22 mmol) in pyridine (25 mL) cooled at 0−5 °C. The mixture was stirred at 0−5 °C for 3 h, then poured in 2 M HCl (200 mL) and extracted with EtOAc (100 mL). The organic phase was washed with satd aq Na_2CO_3 (50 mL), water and brine, dried with anhydrous sodium sulfate, and concentrated under vacuum. The title compound (3.75 g, 84% yield) was obtained as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 9.80 (bs, 1H), 7.59 (m, 2H), 7.27 (m, 2H), 7.01 (m, 1H), 2.33−2.20 (m, 3H), 1.78−1.48 (m, 6H), 1.21−1.16 (m, 2H). MS (ESI) m/z 204 [$(M + H)^+$].

By analogous procedure, starting from cyclohexylacetic acid, the following compound was prepared:

2-Cyclohexyl-N-phenyl-acetamide (General Formula G, Scheme 3). Yield 2.38 g, 90%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 9.80 (bs, 1H), 7.59 (m, 2H), 7.29 (m, 2H), 7.01 (m, 1H), 2.18 (d, J = 7.08 Hz, 2H), 1.76 (m, 1H), 1.74−1.65 (m, 4H), 1.22−1.12 (m, 4H), 1.02−0.92 (m, 2H). MS (ESI) m/z 218 [$(M + H)^+$].

2-Cyclopentyl-N-phenyl-thioacetamide (General Formula H, Scheme 3). To a solution of 2-cyclopentyl-*N*-phenyl-acetamide (3.70 g, 18.23 mmol) in toluene (80 mL), phosphorus pentasulfide (4.46 g, 20.05 mmol) was added. The mixture was heated at 70 °C for 2 h, then was cooled at room temperature and filtered on Celite. After evaporation under vacuum, the crude residue was purified by silica gel column chromatography (exane/EtOAc 85:15) to afford the title compound (1.54 g, 39% yield) as yellow oil. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 11.43 (bs, 1H), 7.77 (m, 2H), 7.41 (m, 2H), 7.23 (m, 1H), 2.75 (d, J = 7.45 Hz, 2H), 2.43 (m, 1H), 1.77−1.52 (m, 6H), 1.26−1.23 (m, 2H). MS (ESI) m/z 220 [$(M + H)^+$].

By analogous procedure, starting from 2-cyclohexyl-*N*-phenyl-acetamide, the following compound was prepared:

2-Cyclohexyl-N-phenyl-thioacetamide (General Formula H, Scheme 3). Yield 0.96 g, 38%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 11.41 (bs, 1H), 7.77 (m, 2H), 7.39 (m, 2H), 7.23 (m, 1H), 2.64 (d, J = 7.20 Hz, 2H), 1.96 (m, 1H), 1.75−1.68 (m, 4H), 1.24−1.15 (m, 4H), 1.01−0.90 (m, 2H). MS (ESI) m/z 234 [$(M + H)^+$].

2-Cyclopentyl-N-phenyl-thioacetimidic Acid Methyl Ester (General Formula I, Scheme 3). To a solution of 2-cyclopentyl-*N*-phenyl-thioacetamide (1.54 g, 7.03 mmol) in MeCN (25 mL), methyl iodide (0.86 mL, 14.06 mmol) and cesium carbonate (4.60 g, 14.06 mmol) were added. The mixture was heated at 50 °C for 1 h, then was cooled at room temperature and poured in water (100 mL). The suspension was extracted with EtOAc (2 × 30 mL), then the organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated under vacuum. The title compound (1.51g, 92% yield) was obtained as yellow oil. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.30 (m, 2H), 7.03 (m, 1H), 6.70 (m, 2H), 2.40−2.32 (m, 5H), 1.96 (m, 1H), 1.63−1.12 (m, 6H), 1.04−0.89 (m, 2H). MS (ESI) m/z 234 [$(M + H)^+$].

By analogous procedure, starting from 2-cyclohexyl-*N*-phenyl-thioacetamide, the following compound was prepared:

2-Cyclohexyl-N-phenyl-thioacetimidic Acid Methyl Ester (General Formula I, Scheme 3). Yield 0.85 g, 85%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.30 (m, 2H), 7.03 (m, 1H), 6.69 (m, 2H), 2.33−2.25 (m, 5H), 1.92 (m, 1H), 1.63−1.45 (m, 4H), 1.24−1.00 (m, 4H), 0.75−0.64 (m, 2H). MS (ESI) m/z 248 [$(M + H)^+$].

*3-Cyclopentylmethyl-5-phenoxymethyl-4-phenyl-4H-[1,2,4]-triazole (**61**).* A mixture of 2-cyclopentyl-*N*-phenyl-thioacetimidic acid methyl ester (0.30 g, 1.29 mmol) and phenoxy-acetic acid hydrazide (0.21 g, 1.29 mmol) in DMF (5 mL) was heated at 120 °C for 7 h, then was cooled at room temperature, poured in water (50 mL), and extracted with EtOAc (2 × 20 mL). The organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated under vacuum. The crude residue was purified by silica gel column chromatography (DCM/MeOH 95:5) to afford the title compound (0.11 g, 25% yield) as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.57−7.52 (m, 3H), 7.48−7.45 (m, 2H), 7.22 (m, 2H), 6.92 (m, 1H), 6.85 (m, 2H), 5.02 (s, 2H), 2.55 (d, J = 7.45 Hz, 2H), 2.06 (m, 1H), 1.63 (m, 2H), 1.52−1.40 (m, 6H). MS (ESI) m/z 334 [$(M + H)^+$]. HRMS (ESI) calculated for $C_{21}H_{24}N_3O^+$ [$(M + H)^+$] 334.1914; found 334.1916.

By analogous procedure, starting from 2-cyclohexyl-*N*-phenyl-thioacetimidic acid methyl ester, the following compound was prepared:

*3-Cyclohexylmethyl-5-phenoxymethyl-4-phenyl-4H-[1,2,4]-triazole (**62**).* Yield 0.11 g, 26%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 7.57−7.52 (m, 3H), 7.46−7.44 (m, 2H), 7.22 (m, 2H), 6.92 (m, 1H), 6.85 (m, 2H), 5.02 (s, 2H), 2.44 (d, J = 6.71 Hz, 2H), 1.63−1.47 (m, 6H), 1.12−1.05 (m, 3H), 0.87−0.79 (m, 2H). MS (ESI) m/z 348 [$(M + H)^+$]. HRMS (ESI) calculated for $C_{22}H_{26}N_3O^+$ [$(M + H)^+$] 348.2071; found 348.2069.

*3-[3-(2-Chloro-phenoxymethyl)-5-cyclopentylsulfanyl-[1,2,4]-triazol-4-yl]-pyridine (**67**).* To a solution of **14** (0.05 g, 0.17 mmol) in anhydrous DMF (2 mL), 2-chlorophenol (0.02 g, 0.18 mmol) and potassium carbonate (0.05 g, 0.34 mmol) were added. The mixture was heated at 80 °C for 1 h, then was cooled at room temperature and poured in water (20 mL). The suspension was extracted EtOAc (2 × 20 mL), the organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated under vacuum. The crude residue was purified by silica gel column chromatography (DCM/MeOH 98:2) to afford the title compound (0.05 g, 76% yield) as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.72−8.70 (m, 2H), 8.00 (m, 1H), 7.60 (m, 1H), 7.36 (m, 1H), 7.28−7.19 (m, 2H), 6.96 (m, 1H), 5.26 (s, 2H), 3.83 (m, 1H), 2.07 (m, 2H), 1.66−1.54 (m, 6H). MS (ESI) m/z 387 [$(M + H)^+$]. HRMS (ESI) calculated for $C_{19}H_{20}ClN_4OS^+$ [$(M + H)^+$] 387.1041; found 387.1042.

By analogous procedure, starting from **14** and the appropriate substituted phenol, derivatives **68**−**74**, **76**−**80**, **82**, **84**, **85**, **87**−**89** (see Supporting Information, p S13), and the following compounds were prepared:

*3-[3-(4-Bromo-phenoxymethyl)-5-cyclopentylsulfanyl-[1,2,4]-triazol-4-yl]-pyridine (**75**).* Yield 0.07 g, 96%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.70 (m, 1H), 8.67 (m, 1H), 7.98 (m, 1H), 7.59 (m, 1H), 7.25 (m, 2H), 6.85 (m, 2H), 5.13 (s, 2H), 3.80 (m, 1H), 2.07 (m, 2H), 1.65−1.51 (m, 6H). MS (ESI) m/z 431 [$(M + H)^+$]. HRMS (ESI) calculated for $C_{19}H_{20}BrN_4OS^+$ [$(M + H)^+$] 431.0536; found 431.0534.

*4′-(5-Cyclopentylsulfanyl-4-pyridin-3-yl-4H-[1,2,4]triazol-3-ylmethoxy)-biphenyl-4-carbonitrile (**81**).* Yield 0.06 g, 79%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.73−8.71 (m, 2H), 8.03 (m, 1H), 7.88 (m, 2H), 7.82 (m, 2H), 7.68−7.60 (m, 3H), 7.00 (m, 2H), 5.22 (s, 2H), 3.82 (m, 1H), 2.06 (m, 2H), 1.66−1.54 (m, 6H). ^{13}C NMR (100.7 MHz), δ (ppm, DMSO-d_6): 157.7, 152.0, 151.5, 150.9, 147.7, 143.9, 135.2, 132.7, 131.4, 129.9, 128.2, 126.9, 124.3, 118.8, 115.3, 109.3, 60.0, 46.0, 33.1, 24.0. MS (ESI) m/z 454 [$(M + H)^+$]. HRMS (ESI) calculated for $C_{26}H_{24}N_5OS^+$ [$(M + H)^+$] 454.1696; found 454.1688. Melding point 144−145 °C.

*3-[3-(4-Bromo-3-methyl-phenoxymethyl)-5-cyclopentylsulfanyl-[1,2,4]triazol-4-yl]-pyridine (**83**).* Yield 0.07 g, 86%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.73 (m, 1H), 8.69 (m, 1H), 8.00 (m, 1H), 7.61 (m, 1H), 7.41 (d, J = 8.79 Hz, 1H), 6.86 (d, J = 2.81 Hz, 1H), 6.65 (m, 1H), 5.12 (s, 2H), 3.81 (m, 1H), 2.25 (s, 3H), 2.06 (m, 2H), 1.62−1.54 (m, 6H). MS (ESI) m/z 445 [$(M + H)^+$]. HRMS (ESI) calculated for $C_{20}H_{22}BrN_4OS^+$ [$(M + H)^+$] 445.0692; found 445.0683.

*3-[3-(4-Chloro-3-methyl-phenoxymethyl)-5-cyclopentylsulfanyl-[1,2,4]triazol-4-yl]-pyridine (**86**).* Yield 0.06 g, 88%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.73 (m, 1H), 8.69 (m, 1H), 8.00 (m,

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1H), 7.62 (m, 1H), 7.26 (d, J = 8.79 Hz, 1H), 6.85 (d, J = 3.05 Hz, 1H), 6.72 (m, 1H), 5.13 (s, 2H), 3.82 (m, 1H), 2.24 (s, 3H), 2.05 (m, 2H), 1.66−1.50 (m, 6H). ^{13}C NMR (100.7 MHz), δ (ppm, DMSO-d_6): 155.9, 151.9, 151.4, 150.8, 147.7, 136.4, 135.2, 129.9, 129.4, 125.5, 124.3, 117.4, 113.9, 60.1, 46.0, 33.1, 24.0, 19.6. MS (ESI) m/z 401 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{20}H_{22}ClN_4OS^+$ [(M + H)$^+$] 401.1198; found 401.1202. Melting point 116−117 °C.

*3-{3-Cyclopentylsulfanyl-5-[4-(4,4,5,5-tetramethyl-[1,3,2]-dioxaborolan-2-yl)-phenoxymethyl]-[1,2,4]triazol-4-yl}-pyridine (**90**).* Yield 0.14 g, 58%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.71 (m, 1H), 8.68 (m, 1H), 7.99 (m, 1H), 7.60 (m, 1H), 7.55 (m, 2H), 6.85 (m, 2H), 5.17 (s, 2H), 3.81 (m, 1H), 2.06 (m, 2H), 1.66−1.51 (m, 6H), 1.26 (s, 12H). MS (ESI) m/z 478 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{25}H_{32}BN_4O_3S^+$ [(M + H)$^+$] 478.2319; found 478.2319.

*4′-(5-Cyclopentylsulfanyl-4-pyridin-3-yl-4H-[1,2,4]triazol-3-ylmethoxy)-biphenyl-4-carboxylic Acid Methoxyamide (**91**).* To a solution of **90** (0.07 g, 0.15 mmol) and 4-bromo-*N*-methoxybenzamide (0.04 g, 0.15 mmol) in a mixture of dioxane (3 mL) and water (1 mL), cesium carbonate (0.14 g, 0.44 mmol) and Pd(dppf)Cl$_2$·DCM (10% mol, 0.012 g, 0.015 mmol) were added. The mixture was heated, under argon, by microwave irradiation at 100 °C for 3 h, then cooled at room temperature and poured in water (20 mL). The suspension was extracted with EtOAc (2 × 20 mL), the organic phase was washed with brine, dried with anhydrous sodium sulfate, and evaporated under vacuum. The crude residue was purified by silica gel column chromatography (DCM/MeOH 95:5) to afford the title compound (0.03 g, 40% yield) as white solid. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 11.73 (bs, 1H), 8.74−8.71 (m, 2H), 8.02 (m, 1H), 7.80 (m, 2H), 7.71 (m, 2H), 7.64−7.60 (m, 3H), 6.97 (m, 2H), 5.21 (s, 2H), 3.82 (m, 1H), 3.71 (s, 3H), 2.06 (m, 2H), 1.65−1.52 (m, 6H). MS (ESI) m/z 502 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{27}H_{28}N_5O_3S^+$ [(M + H)$^+$] 502.1908; found 502.1911.

By analogous procedure, starting from **90** or **75** or **83** and the appropriate aryl bromide or aryl boronic acid, derivatives **92**−**115**, **117**, **118** (see Supporting Information, p S17), and the following compound were prepared:

*3-[3-Cyclopentylsulfanyl-5-(4′-methanesulfonyl-2-methyl-biphenyl-4-yloxymethyl)-[1,2,4]triazol-4-yl]-pyridine (**116**).* Yield 0.06 g, 40%. ^{1}H NMR (400 MHz), δ (ppm, DMSO-d_6): 8.75−8.71 (m, 2H), 8.03 (m, 1H), 7.95 (m, 2H), 7.63 (m, 1H), 7.57 (m, 2H), 7.14 (d, J = 8.30 Hz, 1H), 6.83−6.79 (m, 2H), 5.18 (s, 2H), 3.83 (m, 1H), 3.25 (s, 3H), 2.18 (s, 3H), 2.07 (m, 2H), 1.67−1.53 (m, 6H). ^{13}C NMR (100.7 MHz), δ (ppm, DMSO-d_6): 156.9, 152.0, 151.6, 150.9, 147.8, 145.9, 139.0, 136.3, 135.2, 133.1, 130.6, 129.9, 129.9,126.8, 124.3, 116.7, 112.4, 59.9, 46.0, 43.5, 33.1, 24.0, 20.2. MS (ESI) m/z 521 [(M + H)$^+$]. HRMS (ESI) calculated for $C_{27}H_{29}N_4O_3S_2^+$ [(M + H)$^+$] 521.1676; found 521.1678. mp 134−135 °C.

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.[28] 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 phosphoenolpyruvate 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 stoicometric 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 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). Twenty-four hours after seeding, cells were treated with the compounds to be tested and incubated for 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 3 μL of compound diluted in DMSO were added to 3 mL of the appropriate medium in the well with (0.1% DMSO final concentration) and the plates 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% TritonX-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 20 μg of each sample were 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 Antirabbit from Thermo-Scientific). The detection was performed using SuperSignal west Pico Chemiluminescent substrate from Thermo Scientific.

7. In Vivo Pharmacokinetics. The pharmacokinetic profile of the compounds was investigated in overnight fasted male nude mice following a single dose given intravenously (iv) or orally (po). The compound was formulated in 10% Tween 80 in 5% dextrose (iv) or in 0.5% Methocel (os) as vehicle. A total of six mice were treated (three for each leg). Blood samples of each mouse were collected from the saphenous vein at predose, 0.083, 0.5, 1, 6, and 24 h postdosing following iv dosing, and at predose, 0.25, 0.5, 1, 6, and 24 h following oral dosing. Samples were centrifuged at 10000 rpm for 3 min at 4 °C, and the plasma was stored at −80 °C until analysis. Samples were analyzed byLC/MS/MS technique.

Pharmacokinetic Noncompartmental Data Analysis. The pharmacokinetic parameters were derived by noncompartmental methods using the WinNonlin software program. The highest concentration C_{max} and the time to peak t_{max} were read as the coordinates of the highest observed concentration. The terminal half-life ($t_{1/2,z}$) was calculated by the formula $t_{1/2,z} = \ln(2)/\lambda_z$, where λ_z is the slope of the terminal linear phase of natural-log concentrations vs time curve. The choice of the points on the terminal phase was based

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on visual inspection of the data. The area under the plasma level vs time curve, AUC_{∞}, was calculated by the linear trapezoidal rule up to the last detectable concentration $C(t_z)$ and beyond that time by extrapolation from $C(t_z)$ assuming monoexponential decay, using the following formula: $AUC = AUC(0 - t_z) + C(t_z)/\lambda_z$. The following formulas were applied for the estimate of plasma clearance (CL) and volume of distribution at steady state (Vss). $CL = dose/AUC_{\infty}$; $MRT = AUMC/AUC_{\infty}$; $Vss = CL\ MRT$; $AUMC = AUMC(0 - t_z) + C(t_z) \times t_z/\lambda_z + C(t_z)/\lambda_z^2$, with $AUMC(0 - t_z)$ calculated using the linear trapezoidal rule on $C{\cdot}t$ vs t plots. The oral bioavailability (expressed as percent) was estimated by the ratio of dose-normalized AUC_{∞} values after oral and iv dose.

8. In Vitro ADME and PK. High-throughput solubility, Caco-2 cell permeability, and metabolic stability (human liver microsomes) data were acquired according to previously reported procedures.[29]

9. Visualization of the Structure. The VCP biological assembly, i.e., the functional form of the molecule, was shown to be an hexamer. Such a protein complex can be derived from standard symmetry operations in, for example, PyMOL (the PyMOL Molecular Graphics System, version 1.3r2, Schrödinger LLC) on the *Mus musculus* 3 full length chain structure of p97 (PDB entry 3CF1). The low resolution structure made it necessary to "refine" the structure computationally (the Maestro Protein Preparation Wizard, Schrödinger LLC). In addition to protonation and capping, overlaps and flips of crystallographically equivalent side chains were conservatively optimized. The above procedure provided an all-purpose, best possible starting point for structural analysis and exploratory modeling.

■ ASSOCIATED CONTENT

Supporting Information

Analytical data for additional compounds; ^{13}C NMR spectra for representative compounds; biological data. 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; Ub-Pr, ubiquitin-proteasome; PAL, photo affinity labeling; HCT-116, human colorectal carcinoma-116; Caco-2, adenocarcinoma of the colon-2; C/EBP, CCAAT-enhancer-binding proteins; CHOP, CCAAT/enhancer-binding protein homologous protein; gadd153, growth arrest and DNA damage induced gene-153; MG-132, *N*-(benzyloxycarbonyl)-leucinyl-leucinyl-leucinal; NSF, *N*-ethylmaleimide sensitive fusion protein; SPATA5, spermatogenesis-associated protein 5; VPS4B, vacuolar protein sorting-associated protein 4B; Cdc6, cell division cycle 6; Hsp90, heat shock protein 90

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