European Journal of Medicinal Chemistry 69 (2013) 244–261

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Aggrecanase-2 inhibitors based on the acylthiosemicarbazide zinc-binding group

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ARTICLE INFO

Article history:
Received 11 June 2013
Received in revised form
18 August 2013
Accepted 19 August 2013
Available online 30 August 2013

Keywords:
Acylthiosemicarbazide
Aggrecanase
Library

ABSTRACT

Osteoarthritis is a disabling disease characterized by the articular cartilage breakdown. Aggrecanases are potential therapeutic targets for the treatment of this pathology. At the starting point of this project, an acylthiosemicarbazide was discovered to inhibit aggrecanase-2. The acylthiosemicarbazide Zn binding group is also a convenient linker for library synthesis. A focused library of 920 analogs was thus prepared and screened to establish structure–activity relationships. The modification of the acylthiosemicarbazide was also explored. This strategy combining library design and discrete compounds synthesis yielded inhibitor **35**, that is highly selective for aggrecanases over a panel of metalloproteases and inhibits the degradation of native fully glycosylated aggrecan. A docking study generated binding conformations explaining the structure–activity relationships.

1. Introduction

Proteoglycans are key proteins of the extracellular matrix. Among them, collagen is responsible for tensile strength of cartilage whilst aggrecan is the major cartilage proteoglycan that draws water into the matrix and allowing it to resist compression [1]. Osteoarthritis (OA) is a progressive disease of the joints characterized by the degradation of the articular cartilage. Several proteases have been identified as key mediators of this degradation [2]. Aggrecanases, called ADAMTS-4 and -5 (A Disintegrin and Metalloproteinase with Thrombospondin motifs) were shown to be major metalloproteases involved in the degradation of the extracellular matrix, and in particular aggrecan [3]. In particular, ADAMTS-5 was involved in the degradation of aggrecan in a mouse model [4]. There are currently no disease-modifying drugs for osteoarthritis. Treatment often consists of anti-inflammatory agents [5], pain relief and surgery. In that context, inhibitors of aggrecanases could result in treatments for osteoarthritis.

A few series of inhibitors of aggrecanases (Fig. 1) have been disclosed. These include chelating hydroxamates [6], derived from research on inhibition of matrix metalloproteinases (MMPs), *N*-hydroxyformamides [7], hydroxyquinolines [8] and squaric acid *N*-hydroxylamide amides [9] inhibitors. Other templates like spiroindolines [10] and thioxothiazolidinones [11] or 1,2,4-triazol-3-thiols [12] have been explored. Several series of carboxylic acids

Abbreviations used: EtOAc, ethyl acetate; AcOH, acetic acid; ADAMTS-5, a disintegrin and metalloproteinase with thrombospondin motifs 5; Boc, *tert*-butoxycarbonyl; CH_3CN, acetonitrile; DCE, dichloroethane; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMP, Dess–Martin periodinane; DMSO, dimethylsulfoxide; EDCI, *N*-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Et_3N, triethylamine; EtOH, ethanol; HOBt, *N*-hydroxybenzotriazole; MeOH, methanol; OA, osteoarthritis; PBS, phosphate buffered saline; PTSA, para-toluenesulfonic acid; rt, room temperature; SAR, structure–activity relationship; TACE, tumor necrosis factor-α-converting enzyme; THF, tetrahydrofuran; TMB, 3,3′,5,5′-tetramethylbenzidine.

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http://dx.doi.org/10.1016/j.ejmech.2013.08.027

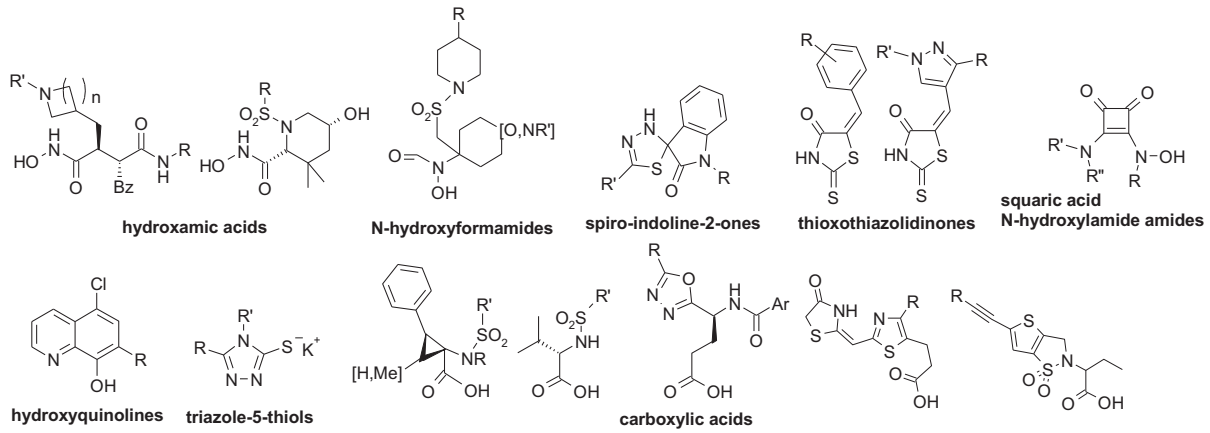


Fig. 1. Zing-binding templates found in published aggrecanase inhibitors.

with various potencies were recently published [13]. A series of selective triazine-based inhibitors, devoid of a zinc-chelating group was recently disclosed, and its binding site remains unknown [14].

In the course of the synthesis of 1,2,4-triazole-3-thiols which were found as inhibitors of ADAMTS-5 [12], we evaluated the activity of one synthetic intermediate acylthiosemicarbazide against aggrecanase-2. Surprisingly, this compound was found to be inhibitor in the micromolar range (Fig. 2). Based on the 3D-structure of co-crystals of inhibitors and aggrecanase [15] we hypothesized that more flexible linker like acylthiosemicarbazide would be worth exploring. Docking of compound **1** into the binding pocket of ADAMTS-5 (pdb code 3B8Z) suggests that while the sulfur atom interacts with the zinc ion [16], the biphenyl moiety fits well in the shallow part of the S′1 pocket (residues 437–451) (Fig. 3). The chlorophenyl substituents point into the S1 pocket. Also, the carbonyl of the acylthiosemicarbazide accepts a hydrogen bond from Thr378 of the S′2 pocket (residues 371–379). An additional hydrogen bond occurs between NHCO and Glu411 side chain (Fig. 3).

We report here the structure–activity relationships that were driven by the synthesis, screening and biological activities of a 920-member library of acylthiosemicarbazides and discrete analogs. In parallel, changes in the linker were made to complete the structure–activity study. Activities of key compounds were also analyzed in the light of a docking study. The best inhibitor **35** was shown to be selective on a panel of several related metalloproteases. Finally, compounds **35** and **46** were evaluated for their impact on the hydrolysis of full native aggrecan.

2. Results and discussion

2.1. Design and parallel synthesis of a 920-member library

To explore structure–activity relationships around **1** (Fig. 2), we designed a focused library of acylthiosemicarbazides by carbonylation of hydrazides and amines. Sets of 23 hydrazides (**2a**–**w**) and 40 amines (**3a**–**an**) were selected to explore the structure–activity relationships in the series. The resulting 920 acylthiosemicarbazides were enumerated before synthesis and we calculated key structural parameters: number of hydrogen bond donors or acceptors, Log *P*, polar surface area (PSA), molecular weight (MW) and number of rotatable bonds (Table 1) [17].

Hydrazides were derived from phenylacetic and phenoxyacetic acids. The aryl substituents were chosen to study the influence of electronic and steric properties. Hydrazides **2a**–**n** were obtained from the corresponding esters **4a**–**n** by reaction with hydrazine

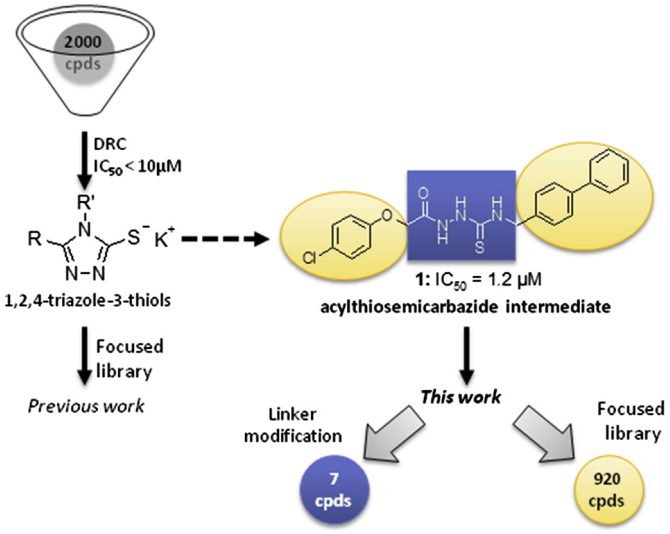


Fig. 2. Discovery of **1** and strategy to optimize acylthiosemicarbazide series.

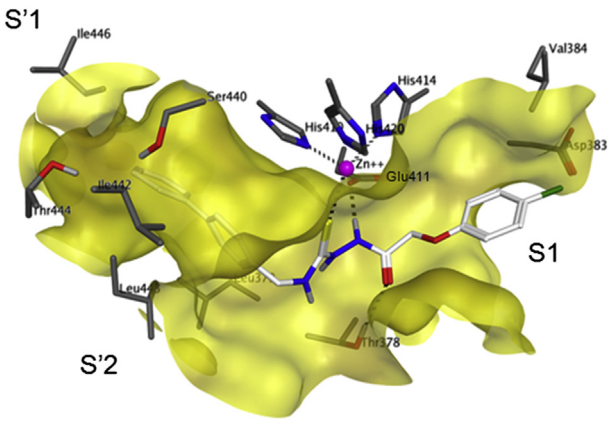


Fig. 3. Putative binding mode for compound 1The inhibitor was docked in the crystallographic structure of ADAMTS-5 (pdb code 3B8Z). Ligand **1** carbon atoms are displayed in white, and key binding site residues as gray sticks. The catalytic zinc ion (magenta) is shown as a sphere. Coordinating and hydrogen bonds are represented as black dotted lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Calculated properties of the library members.[a]

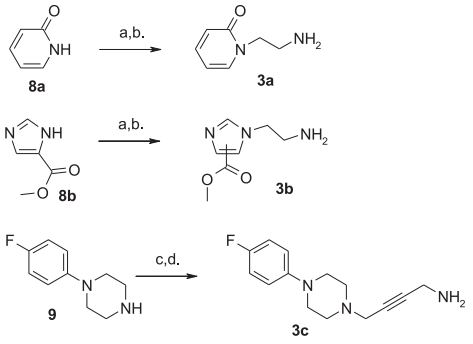
	Mean	Std dev	Target range
MW (g/mol)	408.1	39.6	[350–500]
clog P	3.7	1.3	<5
H Bond Donors	3.1	0.4	<5
H Bond Acceptors	4.0	1.2	<10
Rotatable Bonds	9.5	1.4	<10
PSA (Å^2)	111.1	21.0	[90–140]

[a] Generated by Pipeline Pilot from Accelrys.

hydrate in refluxed ethanol (Scheme 1). In the phenoxy R_1 series, ester precursors were synthesized from the corresponding phenol or aniline by reaction with either ethylbromoacetates **4a**–**g**, **i** or ethyl 2,3-dibromopropanoate for benzoxazine **4j**. Esters **4h** and **4k** were obtained from the corresponding carboxylic acids by reaction with thionyl chloride and ethanol. Finally, hydrazide **2o** was obtained from the corresponding acylchloride with methylhydrazine (Scheme 1) [18]. Hydrazides bearing a 1,2,4-oxadiazole ring were synthesized as previously described by our team [12]. Hydrazides **2p**–**w** were commercially available.

Amines used were benzylamines or homologues, biphenyl derivatives, cyclohexyl compounds or polar and hydrogen bond-donating amines. Several primary amines bearing a pyridinone ring (**3a**) [13a], an imidazole ring (**3b**) [13b] or *N*-arylpiperazine group (**3c**) [13f] were incorporated in the library by analogy to motives recently found in published inhibitors. Amines **3a** and **3b** were synthesized by *N*-alkylation of **8a**, **b** by *N*-Boc-2-bromoethylamine and subsequent deprotection [19] (Scheme 2). Amine **3c** was synthesized by a copper-catalyzed Mannich reaction between *N*-Boc-propargylamine and piperazine **9** (Scheme 2) [20].

Hydrazides and amines reacted in parallel to give the corresponding acylthiosemicarbazides in 2D barcoded tubes (Matrix) at 15 μmol-scale (Scheme 3) [21]. Di-(2-pyridyl) thionocarbonate (PTC) was preferred as the thiocarbonyl transfer agent over *N*,*N*′-thiocarbonyldiimidazole since the latter gave lower conversions with prototypical reagents. The activation step of the amine was set to last 2 h, to allow less reactive amines to form the isothiocyanate, and to prevent remaining PTC to form the undesired 1,3,4-oxadiazol-2-thione directly from the hydrazide. Heating the reaction media was found to be essential for a total conversion and

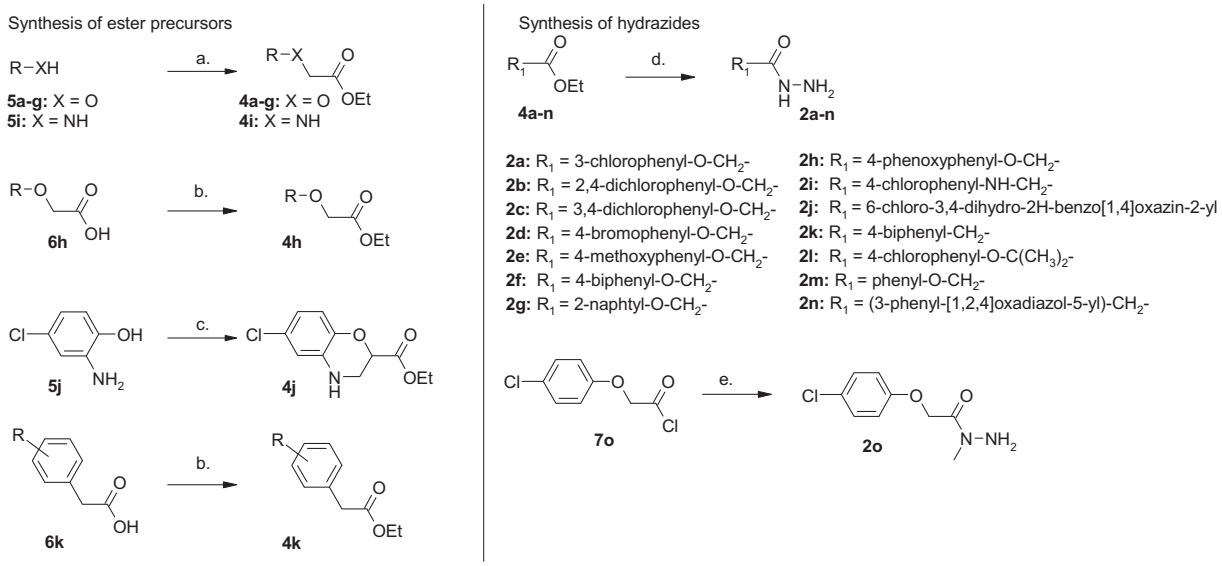


Scheme 2. Synthesis of amines **3a**–**c**.

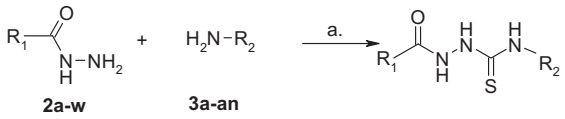
DMF was the best solvent not only for solubilizing all the reagents but also for dispensing solutions with the automated liquid handling system (Tecan Genesis). DMF had to be free of residual dimethylamine and was thus stored overnight on freshly prepared 4 Å molecular sieves before use. Purity of the library was assessed by LC-MS.

2.2. Screening

The library was screened at 30 μM and 3 μM for inhibition of human ADAMTS-5 using a solid-phase bound substrate of 41 amino-acids (see Experimental section). At these concentrations, the focused library displayed hit rates of 32% and 23% respectively (inhibition of *h*ADAMTS-5 higher than 50%). As the library was a complete combinatorial matrix of the reagents engaged, we made an analysis of screening results to draw preliminary trends in structure–activity relationships. No clear trend could be made as a function of the hydrazide properties (R_1 group). We then analyzed the distribution of hits according to the properties of the amine-born moiety (R_2 group). Consistently with docking of **1**, showing the R_2 in the hydrophobic pocket S′1, the impact of lipophilicity on hit-rate is noticeable (Fig. 4). Indeed, a higher number of hits were derived from amines such as biphenylmethyl amines (**3ac**, **3an**, **3l**) and 4-cyclohexylbutylamine **3ag**, in comparison with polar amines such derivatives **3a**, **b**, **3m**, **3j**, which did not give any hits.



Scheme 1. Synthesis of hydrazides **2a**–**o**.



Scheme 3. Synthesis of acylthiosemicarbazides.

2.3. Structure–activity relationships and docking in the thiosemicarbazide series

2.3.1. Variations of R_1 and R_2 substituents

To establish structure–activity relationships in this series, selected compounds were resynthesized at larger scale to allow full analytical characterization and dose–response assays on the enzyme. IC_{50}s in the series range from 0.17 μM to 100 μM.

First, the influence of the nature of the R_1 from the hydrazide substituent was explored (Table 2). Replacement of the chlorine atom by smaller atoms like fluorine (**10**), or hydrogen (**11**) decreased inhibition by 1 or 2-log whereas changing the chlorine position or replacing it by methoxy (**17**), trifluoromethyl (**12**) or nitro (**22**) groups did not impact much activity. Introduction of a second phenyl ring (**18**) in place of a methyl group in **17** had only a small impact on the activity whereas compounds with biphenyl or naphthyl templates on (**19**–**21**) were less active than **1**. Interestingly, substitution of the methylene group in *gem*-dimethyl analog **23** and cyclic derivative **24** led to slightly less active compounds than **1** (IC_{50} 6.9 and 3.3 μM respectively). Noteworthy, introduction of an NH in ortho position of the phenoxy group (**24** vs **1**) was tolerated, whereas isosteric replacement of the oxygen by an NH led to a decrease in activity (**26**). Shortening the chain by directly linking the aromatic ring to the acylthiosemicarbazide motif, increased activity as exemplified by the submicromolar compound **25**.

Table 3 describes the activity of compounds **1**, **27**–**35** deriving from *p*-chlorophenylacetylhydrazide **2q**. In the biphenylmethyl series (**1**, **27**, **28**) ortho-substituted compound **28** was more potent than hit **1** and its meta-substituted analog **27**. Removal of the terminal phenyl ring was deleterious to activity (12 μM vs 1.2 μM for **29** and **1** respectively), while elongation of the methylene linker was beneficial for activity (**29**, **31**, **33**–**34**). As expected from screening, introduction of a more polar ring like pyridine (**30**) lead to poorly active compound. Interestingly, in the longer series, the isosteric replacement of the phenyl ring of **33** by an imidazole ring (**32**) had no impact on activity. On the contrary, replacement of the

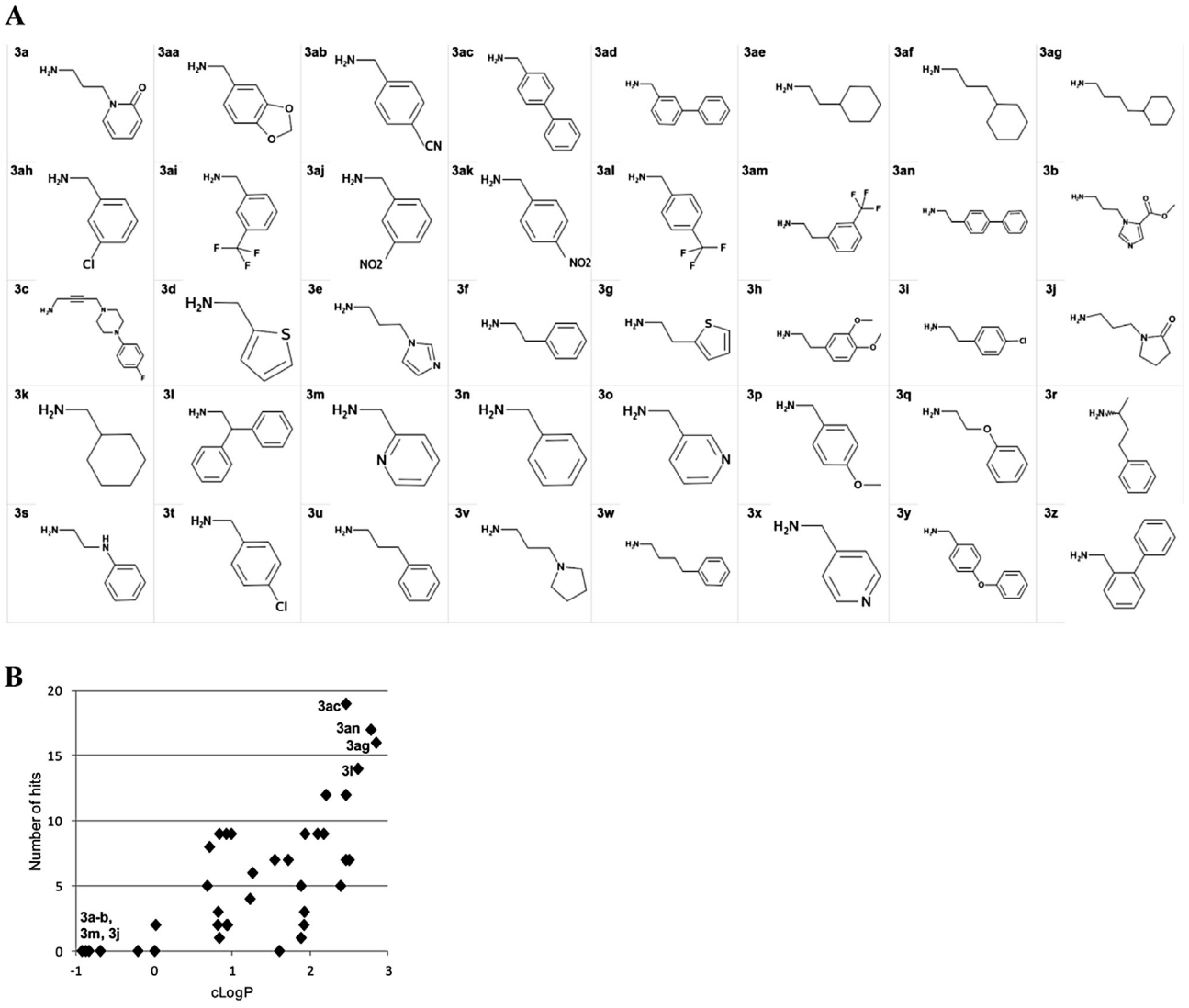
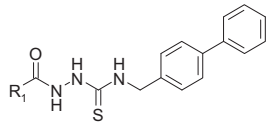
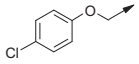
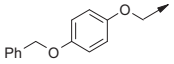
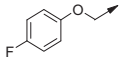
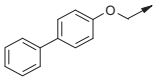
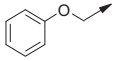
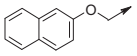
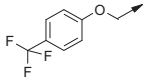
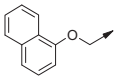
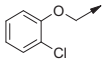
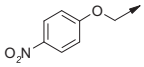
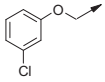
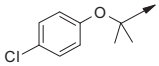
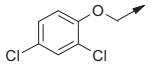
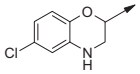
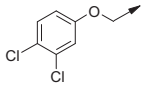
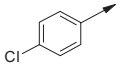
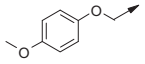
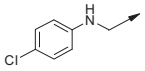


Fig. 4. A) Structures of the 40 amine precursors H_2N-R_2 and B) distribution of hits in function of the *cLog P* of the amine precursors H_2N-R_2.

Table 2
Inhibition of *h*ADAMTS-5 by compounds **1**, **10**–**27**.



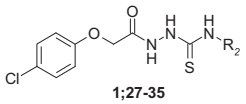
Compd	R_1-	IC_{50}[a] μM (95% CI)	Compd	R_1-	IC_{50}[a] μM (95% CI)
1		1.2 (0.47–2.9)	18		1.0 (0.35–2.5)
10		~100[b]	19		60 (55–82)[c]
11		10 (9.2–11.3)	20		3.2 (2.9–4.1)
12		1.7 (1.5–1.9)	21		4.1 (3.6–4.8)
13		1.9 (1.7–3.5)	22		4.0 (3.8–5.6)
14		2.1 (0.9–3.7)	23		6.9 (5.5–7.9)
15		1.2 (0.71–3.1)	24		3.3 (2.5–4.2)
16		1.2 (1.0–2.2)	25		0.70 (0.51–1.0)
17		1.7 (0.85–3.1)	26		6.3 (5.1–7.2)

[a] Values are means of 2–5 experiments minimum.
[b] 49% inhibition at 100 μM.
[c] Insoluble at 100 μM.

phenyl ring of **33** by a hydrophobic cyclohexyl ring induces a one-log increase in activity (IC_{50} = 0.17 μM for **35**).

Using the knowledge of some of the promising R_1 groups from the first SAR exploration, we combined these with 4-cyclohexylbutyl

Table 3
Inhibition of *h*ADAMTS-5 by compounds **1**, **27**–**35**.



1;27-35

Compd	-R_2	IC_{50}[a] μM (95% CI)
1	-CH_2-*p*-biphenyl	1.2 (0.47–2.9)
27	-CH_2-*m*-biphenyl	2.3 (1.8–2.9)
28	-CH_2-*o*-biphenyl	0.49 (0.32–0.79)
29	-Benzyl	12.6 (4.6–35)
30	-CH_2-3-pyridyl	100[b]
31	-Phenethyl	2.8 (1.9–3.9)
32	–(3-*N*-Imidazolyl)propyl	1.6 (1.2–2.3)
33	–(3-Phenyl)propyl	1.4 (1.0–1.9)
34	–(4-Phenyl)butyl	1.0 (0.6–1.6)
35	–(4-Cyclohexyl)butyl	0.17 (0.16–0.20)

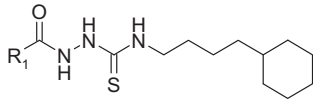
[a] Values are means of 2–5 experiments.
[b] 50% inhibition at 100 μM.

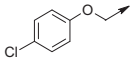
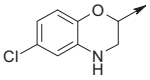
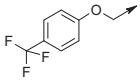
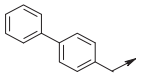
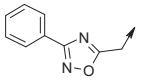
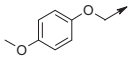
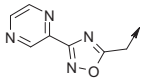
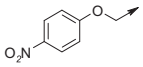
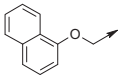
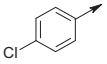
to see if the SAR were additive (Table 4). In the phenoxy R_1 series (**35**–**41**), best inhibitors bear a large, rather hydrophobic substituent while methoxy and unsubstituted analogs (**37**, **38**) are inactive. Cyclic analog **41** is one log less active than its analog **35**. This constrain had a much greater impact in these series than in the biphenyl series (Table 1). A biphenyl R_1 group in combination with 4-cyclohexylbutyl gave the second most active compounds in the series. This compound was highly lipophilic so we evaluated the replacement of one or both rings of the biphenyl moiety by heterocycles. The isosteric replacement of the phenyl ring by an oxadiazole has no impact on activity (**43** vs **42**), while the introduction of a hydrophilic pyrazine reduces the activity by one log (**44** vs **43**). Shortening the chain by removing the phenoxy moiety results in an inactive compound (**45**) whereas removing the methyloxy linker results in the almost equipotent compound **46**. Best inhibitor is the 4-chlorophenoxyacetic derivative **35**.

2.3.2. Docking

The catalytic site of ADAMTS-5 has a funnel shape, which is open at the zinc site and ends up with a L-shaped hydrophobic channel. To better understand the structure–activity relationships on R_2 group brought by the amine precursors, 40 compounds derived from the same hydrazide **2q** (R_1: *para*-chlorophenoxymethyl) and the 40 different amine precursors (**3a**–**3am**, Fig. 3), were docked

Table 4
Inhibition of *h*ADAMTS-5 by compounds **35**–**46**.



Compd	R_1-	IC_{50}[a] μM (95% CI)	Compd	R_1-	IC_{50}[a] μM (95% CI)
35		0.17 (0.16–0.20)	**41**		1.26 (1.1–1.7)
36		0.54 (0.40–0.63)	**42**		0.30 (0.25–0.40)
37		>100[b]	**43**		0.34 (0.27–0.43)
38		>100[c]	**44**		3.10 (2.1–4.6)
39		0.81 (0.63–1.0)	**45**	CH_3-	>100[b]
40		0.88 (0.63–1.3)	**46**		0.67 (0.55–0.81)

[a] Values are means of 2–5 experiments.
[b] Below 20% inhibition at 100 μM.
[c] 50% inhibition at 100 μM.

into the binding pocket of ADAMTS-5 (pdb code 3B8Z) (Fig. 5). Both *cis* and *trans* isomers at each side of the thiourea were docked as both forms exist in solution with little difference in energy [22].

When R_2 is long, linear and hydrophobic, it places in the deep hydrophobic pocket S′1, keeping the *para*-chlorophenoxymethyl group in S1. In most cases, illustrated by the binding of **35** (Fig. 5), the thiourea part of the linker is in a *trans–trans* conformation allowing the sulfur to bind the zinc ion, the amide NH to make a hydrogen bond with Glu411 and CO to accept a hydrogen bond from Thr378. The better interaction of the more flexible cyclopropyl group of **35** in the S′1 hydrophobic pocket, in comparison to other R_2 groups, results in an additional T-stacking interaction between the *para*-chlorophenoxy group and His373 (Fig. 5), accounting for the higher potency of this compound.

Our modeling suggests that when the R_2 group is too large, and so unable to fit in the S′1 pocket for steric reasons, the binding reverses: the chloro-phenoxysubstituent (R_1 group) goes then into the hydrophobic pocket S′1 (compounds **27**, **28**, Fig. 5). This is made possible by the thiourea adopting the less favored *cis–cis* configuration.

2.4. Variations of the putative carbazide zinc binding group

2.4.1. Synthesis of analogs with a modified zinc binding group

To explore the role of the acylthiosemicarbazide moiety in binding, we synthesized several analogs of **1**, **33**–**35** with a modified linker (compounds **47**–**54**, Scheme 4). First, *N*-methylated analogs **47**, **48** were obtained from hydrazide **2o** using the classical procedure depicted in Scheme 3. Analog **49**, where the terminal nitrogen brought by the hydrazide was replaced by a methylene group, was obtained in three steps (Scheme 4). First amine **3ac** was acylated by Boc-glycine. Then the amide was reacted with the Lawesson's reagent to give the thioamide. Subsequent deprotection by TFA and coupling with (4-chlorophenoxy)-acetyl chloride gave **49**. Cyclic derivative **50** was obtained directly from **1** by reaction with chloroacetaldehyde followed by dehydration with TFA. Analogs **51**, **52**, were derived of secondary amines and hydrazide **2q**. Compound **53** has an "inverted" linker where the thiocarbonyl function is closer to the 4-chlorophenyl ring. It was synthesized from hydrazide **2k** and amine **3i**.

Thiol derivative **54** was designed to evaluate the role of the oxidation state of sulfur in the binding. To obtain a stable thiol analog, the linker had to be homologated. Regarding the distance between the chlorophenyl ring and the second phenyl ring, **54** is a direct analog of **33**. First, thiomalic acid reacted with hexafluoroacetone in DMSO to give the 5-oxo-1,3-oxathiolane protected compound [23]. The remaining carboxylic acid function was then converted to the corresponding isothiocyanate using successively $SOCl_2$ and trimethylsilylazide in refluxed toluene. The isothiocyanate reacted with 4-chlorophenoxyacetic acid to give the mixed anhydride that eliminates carbon dioxide upon heating to give the corresponding amide. Finally, the electrophilic 5-oxo-1,3-oxathiolane reacted with phenethylamine to yield **54** (Scheme 4).

2.4.2. Structure–activity relationships for the zinc-binding group

Activities of **47**–**54**, analogs of **1**, **33**–**35**, bearing a different linker, are displayed in Table 5. The nitrogens of the thiosemicarbazide linker are not equivalent. Indeed, substitution on CONH abolishes activity (**47** vs **1** and **48** vs **35**) while substitution at the nitrogen brought by the amine (CSNH) is tolerated (**51**, **52** vs **34**). This is consistent with the binding of CONH with Glu411. Also, isosteric replacement of a nitrogen by $-CH_2-$ (**49**) resulted in

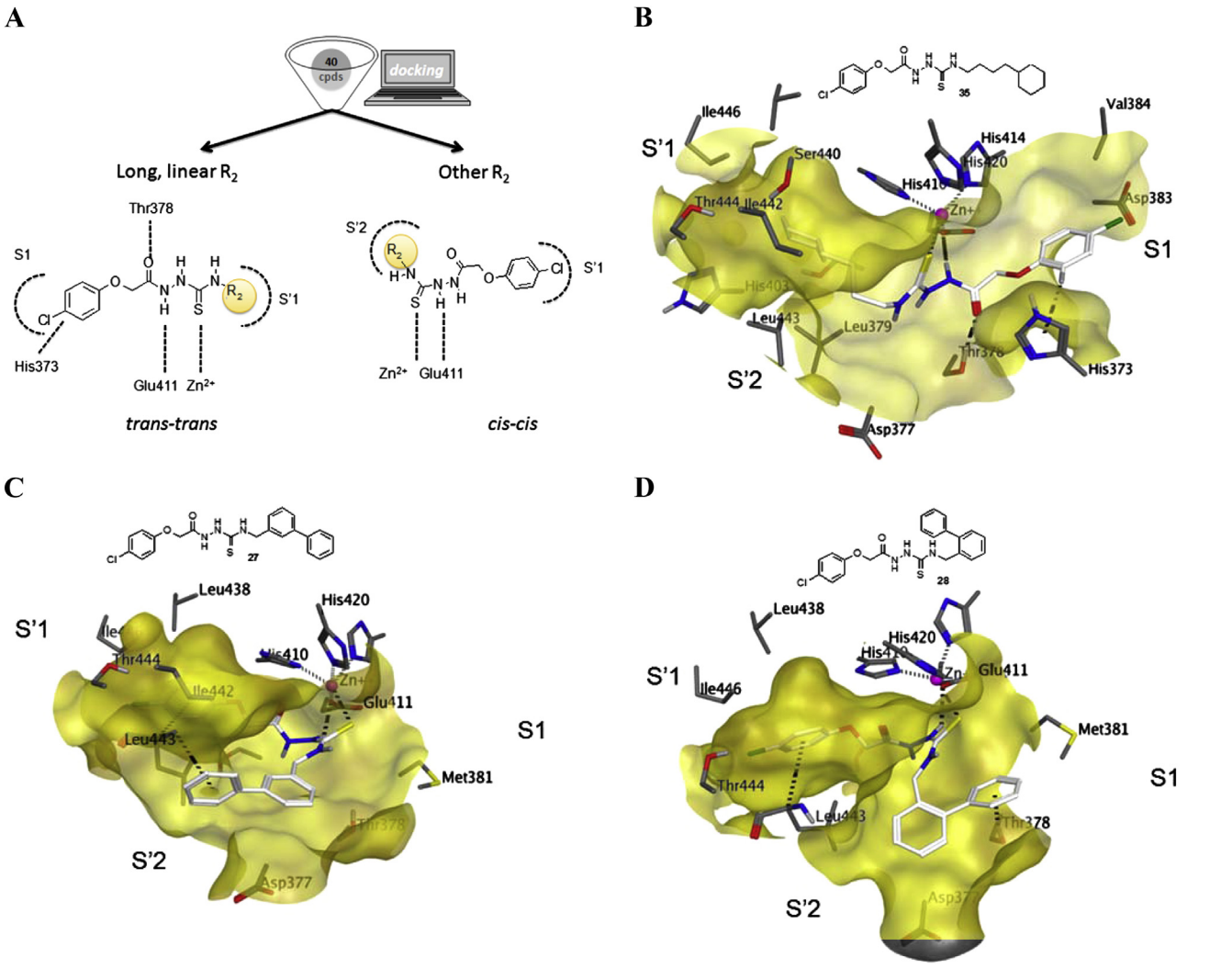


Fig. 5. Putative Binding modes of compounds **35** and **27**–**28** (A) The 40 inhibitors derived of **2q** were docked in X-ray structure of ADAMTS-5 (pdb code 3B8Z). Ligand carbon atoms are displayed in white, and key binding site residues as gray sticks. The catalytic zinc ion (magenta) is shown as a sphere. Coordinating, hydrogen and π–H bonds are represented as black dotted lines. (B) **35**, (C) **27**, (D) **28**. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

complete loss of activity. In docking, no binding of sulfur to zinc is observed. This could be due to the lower basicity of the thioamide versus the thiourea [24]. In compound **50**, the substituent of the thioimidazole mimicks the *trans–trans* configuration of the thiourea. However, docking showed that due to steric hindrance, the hydrogen bond to Glu411 is lost and the binding to zinc ion is weakened resulting in an inactive compound.

Analog **53** is less active than **1**. This can be attributed to both the inversion of the linker that might shift the compound in the pocket of the enzyme and/or the isosteric replacement of the phenoxy group by a phenethyl group.

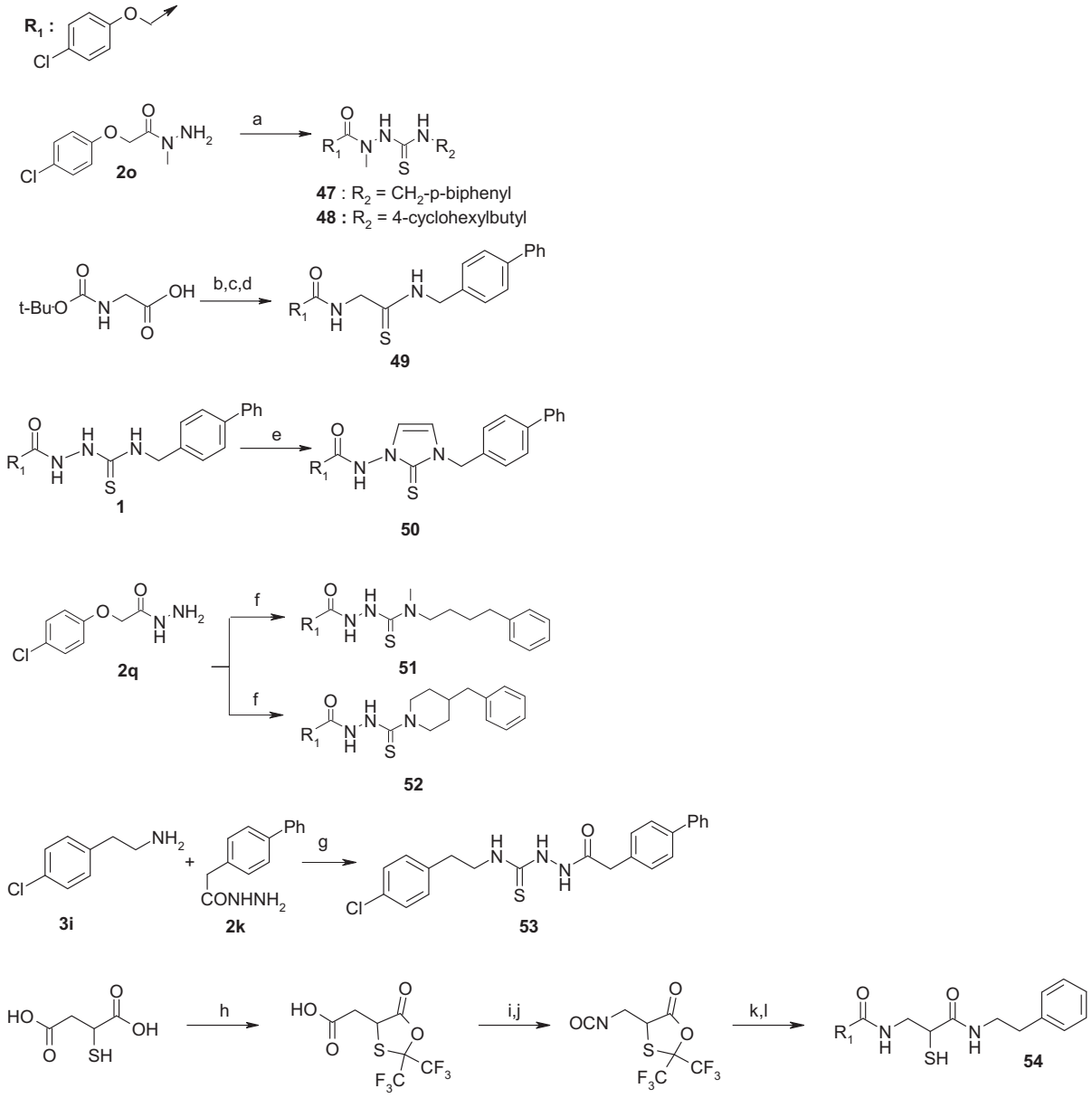
No increase in inhibition was achieved with the thiol derivative **54** as compared to **33**. This could be attributed to the fact that the linker was elongated and both nitrogens in alpha of the thione group were moved or removed.

2.5. Summary of SAR

Fig. 6 summarizes the structure–activity relationships in these series that led to the identification of **35**. The CONH of the thiosemicarbazide function is essential for activity as it makes hydrogen bonds with Glu411 and Thr378 explaining while methylation at this position leads to inactive compounds. On the contrary *N*-substitution at CS–NH–R_1 is tolerated. Long, flexible and hydrophobic groups (R_2) are preferred in S′1. In the phenoxy R_1 series, electronegative substituents on the phenoxy ring R_1, are preferred to interact better with His373. R_1 chain reduction, yielding more compact compounds, is also allowed.

2.6. Activities on related proteases

To further characterize the series, several compounds were assayed for their activity against ADAMTS-4 (Table 6). Though ADAMTS-5 has been shown to be involved in the disease progression in mice [4,25] the relative contribution of ADAMTS-4 and -5 in the human pathology is not fully defined. Recently, ADAMTS-4 levels were shown to be higher in early-compared with middle- or late-stage OA in humans [26]. Interestingly, in our series, all compounds inhibit the two major enzymes responsible for aggrecan cleavage at the Glu^{373}-Ala^{374} site, with little selectivity. This is consistent with the fact that, given the differences in 4 amino-acids residues, the S′1 pocket of ADAMTS-4 is larger than the one of ADAMTS-5 [27]. Compounds **35** and **28** are the most selective for ADAMTS-5 versus ADAMTS-4.



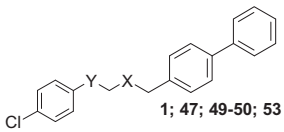
Scheme 4. Synthesis of compounds **47**–**54**.

Compound **35** was then tested in a panel of metalloproteases and was found selective of aggrecanases versus matrix metalloproteases (MMPs) or TNF-α-converting enzyme (TACE) (Table 7). This is consistent with the fact that the S′1 pocket, is defined in the metalloproteinase family, as the specificity pocket [26]. Indeed, in ADAMTS-5, there is a compact S′1 loop counting for half of the pocket [26] while in MMPs the S′1 pocket is mostly entirely enclosed in the S′1 loop. This difference result in decreased activity on MMPs for compounds displaying a longer P′1 group [13f]. Importantly, in ADAMTS-5, the Thr378 residue interacting with the acyl group of our series is replaced by a nonpolar aminoacid in most MMPs.

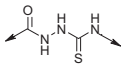
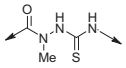
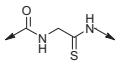
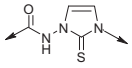
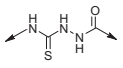
2.7. Inhibition of full native aggrecan hydrolysis

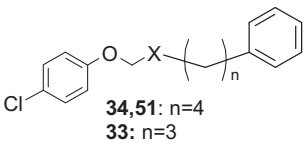
To better assess the inhibitory potency of our compounds, we used an assay where the substrate is full native aggrecan from bovine articular cartilage [28]. Indeed, although we used a longer peptide than usually described in the literature (41 amino-acids versus 8–10 amino-acids), artificial substrate may not interact with potential exosite of the enzyme in the same manner as the native substrate [29] Also, peptidoglycans and cartilage are hydrophilic compartments [30], that may impact the activity of hydrophobic compounds. We thus measured the apparition of the neoepitope ^{374}ARGSVIL, after the action of chondroitinase and keratanase. We tested compound **35** and a less potent analog that however has a lower molecular weight, a more compact structure and a better solubility (**46**). In this assay, both compounds **35** and **46** inhibit in a dose dependent manner ADAMTS-5-mediated hydrolysis of aggrecan from bovine articular cartilage (Fig. 7). Aggrecan is a highly charge polymer in which electrostatic repulsions exert a large influence on its conformation, the viscosity of solutions and diffusion of solutes [31]. The higher inhibition of **46** in the fully glycosylated aggrecan assay, may account for a better compromise between potency and molecular weight.

Table 5
Inhibition of *h*ADAMTS-5 by compounds **1**, **33**, **35** and analogs **47**–**54**.

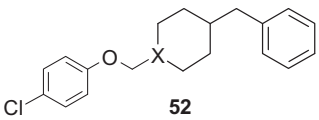


1; 47; 49-50; 53

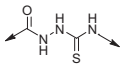
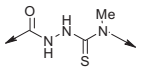
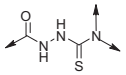
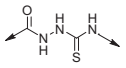
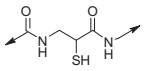
Comtpd	X	Y	IC_{50}[a] μM (95% CI)
1		O	1.2 (0.47–2.9)
47		O	>100[b]
49		O	>100[b]
50		O	>100[b]
53		CH_2	5.2 (3.2–7.9)

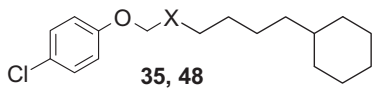


34,51: n=4
33: n=3
54: n=2

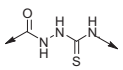
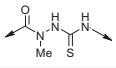


52

Compd	X	IC_{50}[a] μM (95% CI)
34		1.0 (0.6–1.6)
51		1.1 (0.9–1.3)
52		1.6 (1.3–2.0)
33		1.4 (1.0–1.9)
54		8.7 (5.4–13.9)



35, 48

Compd	X	IC_{50}[a] μM (95% CI)
35		0.17 (0.16–0.20)
48		>100[b]

[a] Values are means of 2 experiments minimum.
[b] Less than 30% inhibition at 100 μM.

3. Conclusions

Osteoarthritis (OA) is a pathology of the joints for which no disease-modifying agents are available so far. Proteases implicated in the degradation of aggrecan are potential targets for the treatment of OA. Starting from a novel acylthiosemicarbazide hit, we synthesized a library of 920 compounds where the designed Zn binding group is also a convenient linker between amines and hydrazides. Analysis of structure–activity relationships revealed the key impact of chain length and lipophilicity to fit the S′1 pocket. In particular, the cyclohexylbutyl group was shown to be critical to reach submicromolar activities. Docking studies and activity data from the library and from a set of compounds where the Zn binding linker is modified consistently suggested that the acylthiosemicarbazide makes 3 key interactions in the catalytic site. Compound **35** has an IC_{50} of 170 nM on ADAMTS-5, in the range of most non-hydroxamic aggrecanase inhibitors. Owing to a good fit with the S′1 pocket, **35** is also very selective over other metalloproteases. Also, **35** and **46**, a close, more compact analog of **35** inhibits bovine articular aggrecan hydrolysis by ADAMTS-5.

4. Experimental section

4.1. Biology

4.1.1. ADAMTS-5 inhibition screening assay

Peptide ARGSVILTV-KPIFEVSPSPL(biotinyl)K and peptide QTVTWPDMELPLPRNITEGEARGSVIL-TVKPIFEVSPSPL(biotinyl)K were provided by Eric Diesis (CNRS, UMR8525, Institut de Biologie de Lille, Institut Pasteur de Lille, France). Purified recombinant human ADAMTS-4 and ADAMTS-5 were from Chemicon International (Hampshire, United Kingdom). Aggrecan monomers were purified from bovine nasal cartilage under dissociative conditions according to Hascall and Sajdera [32]. Mouse monoclonal antibody [BC3] to aggrecan ARGxx was from Abcam (Paris, France) and immunoPure goat anti-mouse IgG, peroxidase conjugated was from Pierce Biotechnology (Perbio, Brebières, France). Streptavidin-coated microplates was purchased from Nunc (Roskilde, Denmark). TMB (3,3′,5,5′-tetramethylbenzidine) was from Bio-Rad (Marnes-la-Coquette, France). EDTA and other chemicals were obtained from Sigma–Aldrich (Steinheim, Germany). QTVTWPDMELPLPRNITEGEARGSVIL-TVKPIFEVSPSPL(biotinyl)K, the biotinylated-substrate, was immobilized on streptavidin-coated microplates by adding 100 μL of a 70 nM peptide solution in PBS to each test well and allowing the peptide to bind 2 h at room temperature. The plates were then washed with PBS and dried 30 min at 37 °C. The dried plates were sealed and stored at 4 °C until use. Two wells per plate were coated with the biotinylated-product (ARGSVILTVKPIFEVSPSPL(biotinyl)K) to be used as a quality control for the antibodies detection system. Assays were performed by adding to the previously substrate-coated plates, 100 μL ADAMTS-5 (10 ng/mL) pre-incubated 10 min with test compound or vehicle (1% DMSO) in 50 mM Tris, 5 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij35, pH 7.5. Catalysis was allowed to proceed for 3 h at 37 °C. Reactions were stopped by removing the enzyme solution and washing the wells with PBS 0.05% Tween 20. The primary antibody solution (100 μL of BC-3 antibody at 4.8 ng/mL in PBS, 0.05% Tween 20, 1% BSA) was added to each well and allowed to incubate at room temperature for 1 h. The solution was removed and the wells washed. The HRP-conjugated secondary antibody (100 μL of goat anti-mouse IgG peroxidase conjugated at 96 ng/mL in PBS, 0.05% Tween 20, 1% BSA) was then added in a similar fashion. After washing steps, 100 μL of the peroxidase substrate TMB solution was added to each well. After 15 min the reaction was quenched by addition of 100 μL of 1 N H_2SO_4. Absorbance was then read at 450 nm using a Victor3™ V1420 Perkin Elmer spectrophotometer. EDTA at 50 μM completely abrogated product formation. For screening, the Z and Z′ factors were calculated according to Zhang et al. [33].

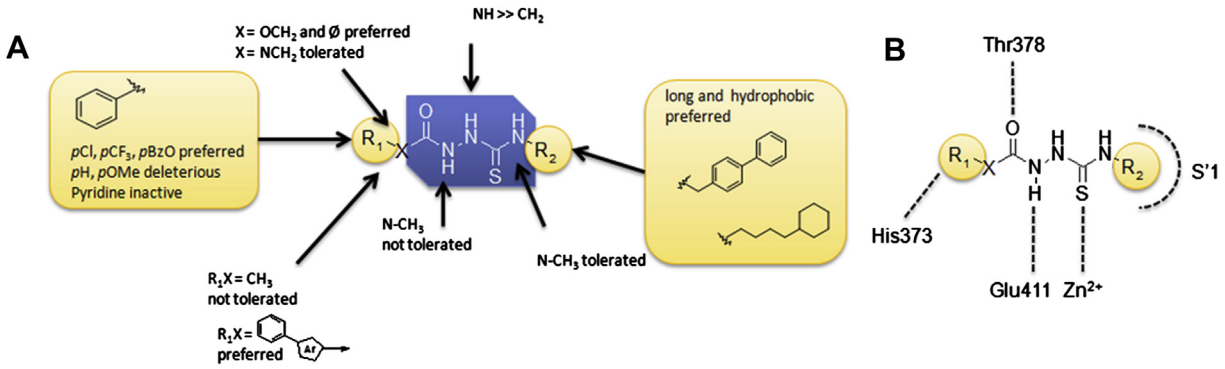


Fig. 6. Summary of SAR in the acylthiosemicarbazide series.

4.1.2. ADAMTS-4 inhibition screening assay

The plates coated with the biotinylated-substrate QTVTWPDMELPLPRNITEGEARGSVILTVKPIFEVSPSPL(biotinyl)K, were prepared as described above. Assays were performed by adding to the previously substrate-coated plates, 100 μL ADAMTS-4 (10 ng/mL) pre-incubated 10 min with test compound or vehicle (1% DMSO) in 50 mM Tris, 5 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij35, pH 7.5. Catalytic reaction was allowed to proceed for 3 h at 37 °C. Reactions were stopped by removing the enzyme solution and washing the wells with PBS 0.05% Tween 20. The primary antibody solution (100 μL of BC-3 antibody at 4.8 ng/mL in PBS, 0.05% Tween 20, 1% BSA) was added to each well and allowed to incubate at room temperature for 1 h. The solution was removed and the wells washed. The HRP-conjugated secondary antibody (100 μL of goat anti-mouse IgG peroxidase conjugated at 96 ng/mL in PBS, 0.05% Tween 20, 1% BSA) was then added in a similar fashion. After washing steps, 100 μL of the peroxidase substrate TMB solution was added to each well. After 15 min the reaction was quenched by addition of 100 μL of 1 N H_2SO_4. Absorbance was then read at 450 nm using a Victor3™ V1420 Perkin Elmer spectrophotometer. EDTA at 50 μM completely abrogated product formation.

4.1.3. ADAMTS-5 inhibition assay of bovine articular aggrecan

3.4 μL of ADAMTS-5 (600 nM) were pre-incubated 20 min at 37 °C with 17 μL test compound or vehicle (4% DMSO) and 40.8 μL of 50 mM Tris buffer, 100 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 0.02% Brij35 at pH 7.5 in a 96-well plate. Bovine articular aggrecan (50 μg/mL final concentration) was then added and the plate was incubated for 48 h at 37 °C. The reaction was stopped by the addition of 17 μL of 200 mM EDTA. A PVDF-membrane plate was conditioned for 5 min with 100 μL of 70% ethanol in water and washed two times with 200 μL of water. 75 μL of the reaction mixture were transferred onto this membrane plate containing 75 μL of 20 mM carbonate/bicarbonate buffer. Samples were left overnight at room temperature. After three washes with 200 μL of PBS 0.05% Tween 20, 100 μL of chondroitinase ABC (10 mU) and keratanase (10 mU) in 50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 0.02% Brij35 at pH 7.5 were added to each well and incubated for 5 h at 37 °C. Wells were washed three times with 200 μL of PBS 0.05% Tween 20 and membranes were blocked with 150 μL of 0.1% milk in PBS 0.05% Tween 20.

The primary antibody solution (100 μL of BC-3 antibody at 2 μg/ml in PBS, 0.05% Tween 20, 0.1% milk) was added to each well and allowed to incubate at room temperature for 1 h. The solution was removed and the wells washed. The HRP-conjugated secondary antibody (100 μL of goat anti-mouse IgG peroxidase conjugated at 0.4 μg/mL in PBS, 0.05% Tween 20, 0.1% milk) was then added in a similar fashion. After washing steps, 100 μL of the peroxidase substrate TMB solution were added to each well. After 20 min the reaction was quenched by addition of 100 μL of 2 N H_2SO_4. 150 μL of this reaction mixture were transferred to a flat-bottomed transparent plate. Absorbance was then read at 450 nm using a Victor3™ V1420 Perkin Elmer spectrophotometer. EDTA at 2 mM completely abrogated product formation.

Table 6
Inhibitory activity on ADAMTS-5 and -4 for selected compounds.

Compd	ADAMTS-5 IC_{50}[a] μM (95% CI)	ADAMTS-4 IC_{50}[a] μM (95% CI)
25	0.70 (0.51–1.0)	1.6 (1.2–1.9)
28	0.49 (0.32–0.79)	2.9 (1.9–3.5)
33	1.4 (1.0–1.9)	2.0 (1.6–2.5)
35	0.17 (0.16–0.20)	0.63 (0.32–1.0)
36	0.54 (0.40–0.63)	0.61 (0.39–0.79)
39	0.81 (0.63–1.0)	0.79 (0.57–1.1)
40	0.88 (0.63–1.3)	1.0 (0.80–1.6)
42	0.30 (0.25–0.40)	0.73 (0.63–0.81)
46	0.67 (0.55–0.81)	1.4 (1.2–1.7)
51	1.1 (0.9–1.3)	1.5 (1.1–2.0)

[a] Values are means of 2 experiments minimum.

4.1.4. Metalloproteases selectivity profile

Profile was performed at CEREP SA as described in Supporting information.

4.2. Molecular modeling

The series of the 40 analogs derived of **2q** and 40 different amine precursors was docked into the binding pocket of ADAMTS-5 using MOE [34]. The protein structure of ADAMTS-5 was downloaded from the Protein DataBank (PDB) under pdb code 3B8Z. Protein protonation was made with the Protonate3D process within MOE 10.2012. The docking used was based on the Induced Fit protocol of MOE, using a Triangle Matcher placement, a first rescoring process using the London dG routine and a force field-based refinement process and a second rescoring process using GBVI/WSA dG keeping in the end the best docking pose for each ligand. No restraints were used during docking but the binding modes were chosen based on both the most favored predicted interaction energy with ADAMTS-5 and a proper chelating of the Zinc ion by the sulfur atom.

4.3. Chemistry

4.3.1. General information

NMR spectra were recorded on a Bruker Avance 300 spectrometer. Chemical shifts are in parts per million (ppm) and were referenced to the residual proton peaks in deuterated solvents.

Table 7
Selectivity profile of compound **35**.

	IC50 (μM) [a]										
Compd	ADAMTS		MMP								TACE
	5	4	1	2	3	7	8	9	12	13	
35	0.17	0.63	>10[b]	>10[b]	>10[b]	>100	47	>10[b]	>100	>100	>10[b]

[a] Values are means of 2 experiments minimum, standard deviations are ±10%.
[b] <10% at 10 μM.

The assignments were made using one dimensional (1D) ^{1}H and ^{13}C spectra (classical or Jmod) and two-dimensional (2D) HSQC, HMBC, ROESY and COSY spectra. For ^{19}F NMR spectra, the chemical shifts were referenced to $CFCl_3$. Mass spectra were recorded with a LC–MS–MS triple–quadrupole system (Varian 1200ws) or a LCMS (Waters Alliance Micromass ZQ 2000). LCMS analysis was performed using a C18 TSK-GEL Super ODS (2 μm particle size column, dimensions 50 mm × 4.6 mm). A gradient starting from 100% H_2O/0.1% formic acid and reaching 20% H_2O/80% CH_3CN/0.08% formic acid within 10 min (method A) or 5 min (method B) at a flow rate of 1 mL/min was used. Preparative HPLC were performed using a Varian PRoStar system using an OmniSphere 10 column C18 250 mm × 41.4 mm Dynamax from Varian, Inc. A gradient starting from 20% CH_3CN/80% H_2O/0.1% formic acid and reaching 100% CH_3CN/0.1% formic acid at a flow rate of 80 mL/min or 20% MeOH/80% H_2O/0.1% formic acid reaching 100% MeOH/0.1% formic acid was used. Purity (%) was determined by reversed phase HPLC, using UV detection (215 nm), and all compounds showed purity greater than 95%; Melting points were determined on a Büchi B-540 apparatus and are uncorrected. All commercial reagents and solvents were used without further purification. Organic layers obtained after extraction of aqueous solutions were dried over $MgSO_4$ and filtered before evaporation under reduced pressure. Purification yields were not optimized. Thick layer chromatography was performed with Silica Gel 60 (Merck, 40–63 μm).

4.3.2. General procedure for the synthesis of hydrazides from corresponding esters (Procedure A)

Ester (1 eq) and $N_2H_2.H_2O$ (5 eq) were solubilized in EtOH (5 mL/mmol). The resulting mixture was refluxed 18–24 h. The reaction was monitored by TLC (CH_2Cl_2/MeOH, 95/5) and LCMS. Solvents were removed under reduced pressure and crude products were extracted with CH_2Cl_2 or ethyl acetate. The combined organics were washed with saturated Na_2CO_3 aqueous solution, and dried (Na_2SO_4) and concentrated under reduced pressure to give the desired hydrazide.

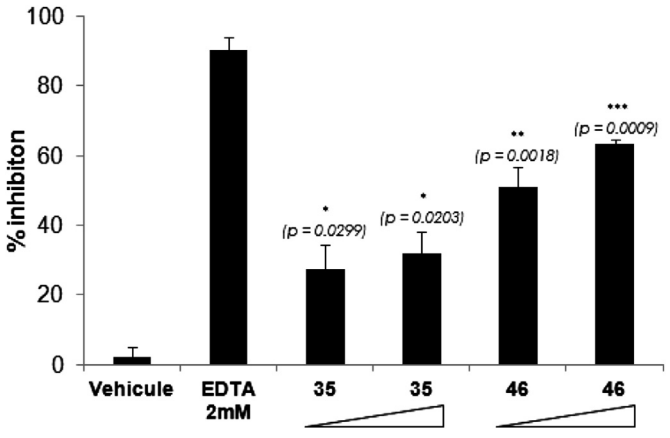


Fig. 7. Inhibition of ADAMTS-5 mediated hydrolysis of aggrecan from bovine articular cartilage by compound **35** and **46**.

4.3.3. General procedure for the synthesis of phenoxyacetic ethyl esters or N-phenylglycine ethyl ester from corresponding phenols or aniline (Procedure B)

Ethyl bromoacetate (1.1 eq) was added to a suspension of the phenol (1 eq) or aniline (1 eq) and potassium carbonate (1.4 eq) in acetone (10 mL). The mixture was refluxed for 20 h. After cooling and evaporation of the solvent, the residue was treated with water and extracted with ethyl acetate. The organic layers were washed with water, dried over $MgSO_4$, and concentrated under reduced pressure to give the corresponding ester.

*4.3.4. 3-Chlorophenoxyacetic acid hydrazide (**2a**)*

3-Chlorophenoxyacetic acid hydrazide (**2a**) was obtained from ester **5a** prepared from the corresponding phenol and ethyl bromoacetate following procedure B. Yield 98%; ^{1}H NMR (300 MHz, CD_3OD): δ (ppm): 1.28 (t, J = 6.7 Hz, 3H), 4.26 (q, J = 6.7 Hz, 2H), 4.72 (s, 2H), 6.85 (ddd, J = 0.8 Hz, J = 2.5 Hz, J = 8.4 Hz, 1H), 6.95–7.00 (m, 2H), 7.26 (dt, J = 7.6 Hz, J = 0.8 Hz, 1H). Ester **4a** was then reacted with hydrazine according to procedure A, to give **2a** as a white powder (98%), ^{1}H NMR 300 MHz ($CDCl_3$): δ (ppm): 4.57 (s, 2H), 6.80 (ddd, J = 0.8 Hz, J = 2.5 Hz, J = 8.4 Hz, 1H), 6.93 (t, J = 2.2 Hz, 1H), 7.03 (ddd, J = 0.8 Hz, J = 2.5 Hz, J = 8.4 Hz, 1H), 7.25 (t, J = 8.2 Hz, 1H) 7.77 (br s, 1H, NH). MS (ESI+) m/z 201 $[M + H]^+$.

*4.3.5. 2,4-Dichlorophenoxyacetic acid hydrazide (**2b**)*

2,4-Dichlorophenoxyacetic acid hydrazide (**2b**) was obtained from ester **5b** prepared from the corresponding phenol and ethyl bromoacetate following procedure B (98%), ^{1}H NMR (300 MHz, CD_3OD): δ (ppm): 1.28 (t, J = 6.7 Hz, 3H), 4.25 (q, J = 6.7 Hz, 2H), 4.80 (s, 2H), 6.97 (d, J = 8.7 Hz, 1H), 7.24 (dd, J = 2.7 Hz, J = 8.7 Hz, 1H), 7.43 (d, J = 2.7 Hz, 1H). Ester **4b** was then reacted with hydrazine according to procedure A, to give **2b** as a white powder, yield 98%, Purity 99%, ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 3.98 (br s, NH), 4.61 (s, 2H), 6.83 (d, J = 8.7 Hz, 1H), 7.23 (dd, J = 2.7 Hz, J = 8.7 Hz, 1H), 7.41 (d, J = 2.7 Hz), 7.94 (br s, 1H, NH), MS (ESI+) m/z 236 $[M + H]^+$.

*4.3.6. 3,4-Dichlorophenoxyacetic acid hydrazide (**2c**)*

3,4-Dichlorophenoxyacetic acid hydrazide (**2c**) was obtained from ester **5c** prepared from the corresponding phenol and ethyl bromoacetate following procedure B (94%). Ester **4c** was then reacted with hydrazine according to procedure A, to give **2c** as a white powder, yield 98%, ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.26 (br s, 2H), 4.54 (s, 2H), 6.98 (dd, J = 3.0 Hz, J = 9.0 Hz, 1H), 7.25 (d, J = 2.9 Hz, 1H), 7.52 (d, J = 9.0 Hz, 1H), 8.93 (br s, NH). MS (ESI+) m/z 236 $[M + H]^+$.

*4.3.7. 4-Bromo-phenoxyacetic acid hydrazide (**2d**)*

4-Bromo-phenoxyacetic acid hydrazide (**2d**) was obtained from ester **5d** prepared from the corresponding phenol and ethyl bromoacetate following procedure B (68%); ^{1}H NMR (300 MHz, CD_3OD): δ (ppm): 1.28 (t, J = 6.7 Hz, 3H), 4.25 (q, J = 6.7 Hz, 2H), 4.69 (s, 2H), 6.77 (d, J = 9.0 Hz, 1H), 7.40 (d, J = 9.1 Hz). Ester **4d** was then reacted with hydrazine according to procedure A, to give **2d** as a white powder, yield 93%, ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.11 (br s, NH_2), 4.48 (s, 2H), 6.92 (d, J = 9.0 Hz), 7.44 (d, J = 9.0 Hz), 9.35 (br s, 1H, NH). MS (ESI+) m/z 246 $[M + H]^+$.

*4.3.8. 4-Methoxy-phenoxyacetic acid hydrazide (**2e**)*

4-Methoxy-phenoxyacetic acid hydrazide (**2e**) was obtained from ester **5e** prepared from the corresponding phenol and ethyl bromoacetate following procedure B (97%), ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.28 (t, J = 6.7 Hz, 3H), 3.78 (s, 3H), 4.24 (q, J = 6.7 Hz, 2H), 4.58 (s, 2H), 6.82–6.90 (m, 4H), MS (ESI+) m/z 211 $[M + H]^+$. Ester **4e** was then reacted with hydrazine according to

procedure A, to give **2e** as a white powder, yield 97% ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 3.78 (s, 3H), 3.94 (br s, NH_2), 4.54 (s, 2H), 6.86 (s, 4H), 7.76 (br s, NH), MS (ESI+) *m*/*z* 246.1 $[M + H]^+$.

*4.3.9. 4-Phenyl-phenoxyacetic acid hydrazide (**2f**)*

4-Phenyl-phenoxyacetic acid hydrazide (**2f**) was obtained from ester **5f** prepared from the corresponding phenol and ethyl bromoacetate following procedure B (85%), ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.33 (t, $J = 6.9$ Hz, 3H), 4.30 (q, $J = 6.9$ Hz, 2H), 4.68 (s, 2H), 6.99 (d, $J = 8.7$ Hz, 2H), 7.33 (t, $J = 6.9$ Hz, 1H), 7.43 (t, $J = 7.8$ Hz, 2H), 7.52–7.58 (m, 4H). Ester **4f** was then reacted with hydrazine according to procedure A, to give **2f** as a white powder, yield 96%, purity 95%, ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.38 (br s, 2H, NH_2), 4.53 (s, 2H), 7.03 (d, $J = 8.7$ Hz, 2H), 7.31 (t, $J = 6.9$ Hz, 1H), 7.42 (t, $J = 7.8$ Hz, 2H), 7.58–7.62 (m, 4H), MS (ESI+) *m*/*z* 243 $[M + H]^+$.

*4.3.10. 2-Naphthyloxyacetylhydrazine (**2g**)*

2-Naphthyloxyacetylhydrazine (**2g**) was obtained from ester **5g** prepared from the corresponding phenol and ethyl bromoacetate following procedure B (92%), ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.30 (t, $J = 7.16$ Hz, 3H), 4.28 (q, $J = 7.16$ Hz, 2H), 4.76 (s, 2H), 7.09 (d, $J = 2.6$ Hz, 1H), 7.24 (dd, $J = 2.39$ Hz, $J_2 = 8.47$ Hz, 1H), 7.34–7.49 (m, 2H), 7.72–7.80 (m, 3H). Ester **4g** was then reacted with hydrazine according to procedure A, to give **2g** as a white powder. Yield 98%; Purity 97%, ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 3.97 (br s, 2H, NH_2), 4.72 (s, 2H), 6.04 (br s, 1H, NH), 7.14–7.19 (m, 2H), 7.40 (t, $J = 6.6$ Hz, 2H), 7.48 (t, $J = 6.6$ Hz, 2H), 7.74–7.82 (m, 3H), MS (ESI+) *m*/*z* 217 $[M + H]^+$.

*4.3.11. 4-Benzyloxyphenoxyacetic acid hydrazide (**2h**)*

A solution of 4-benzyloxyphenoxyacetic acid (1 eq) and thionyl chloride (10 eq) in dry CH_2Cl_2 (10 mL) was stirred for 1 h at room temperature. The reaction mixture was quenched with EtOH and evaporated under reduced pressure to allow quantitative isolation of ethyl ester **4h**. The ester was then reacted with hydrazine according to procedure A, to give **2h** as a white powder. Yield 98%; Purity 100%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 3.93 (br s, 2H), 4.54 (s, 2H), 5.04 (s, 2H), 6.84 (d, $J = 9.3$ Hz, 2H), 6.92 (d, $J = 9.3$ Hz, 2H), 7.32–7.44 (m, 5H), 7.81 (br s, NH), MS (ESI+) *m*/*z* 273 $[M + H]^+$.

*4.3.12. N-(4-Chlorophenyl)glycine hydrazide (**2i**)*

N-(4-Chlorophenyl)glycine hydrazide (**2i**) was obtained from ester **5i** prepared from the corresponding phenol and ethyl bromoacetate following procedure B (95%), ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.32 (t, $J = 6.9$ Hz, 3H), 3.89 (s, 2H), 4.27 (q, $J = 6.9$ Hz, 2H), 6.55 (dd, $J = 6.6$ Hz, $J = 2.1$ Hz, 2H), 7.15 (dd, $J = 6.6$ Hz, $J = 2.1$ Hz, 2H). Ester **4i** was then reacted with hydrazine according to procedure A, to give **2i** as a white powder. Yield 98%; Purity 98%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 3.61 (d, $J = 5.0$ Hz, 2H), 4.20 (br s, 2H, NH_2), 6.09 (t, $J = 5.0$ Hz, 1H), 6.55 (d, $J = 8.7$ Hz, 2H), 7.09 55 (d, $J = 8.7$ Hz, 2H), 9.12 (br s, 1H, NH), MS (ESI+) *m*/*z* 200 $[M + H]^+$.

*4.3.13. 6-Chloro-3,4-dihydro-2H-1,4-benzoxazine-2-carboxylic acid hydrazide (**2j**)*

6-Chloro-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylic acid hydrazide (**2j**) was prepared from 3-chloro-2-aminophenol (1 eq) and ethyl 2,3-dibromopropanoate (1.1 eq) in suspension in acetone. Potassium carbonate (2.8 eq) was added and the mixture was refluxed for 20 h. After cooling and evaporation of the solvent, the residue was treated with water and extracted with ethyl acetate. The combined organic layers were dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified on silica gel (EtOAc/cyclohexane (1:9)) to give ester **4j** as a pale yellow powder. Yield 77%; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.28 (t, $J = 6.9$ Hz, 3H), 3.58 (d, $J = 4.2$ Hz, 2H), 4.13 (dd, $J = 6.9$ Hz, $J = 12$ Hz, 1H), 4.21–4.31 (m, 2H), 4.81 (t, $J = 4.2$ Hz, NH), 6.53 (d, $J = 8.6$ Hz, 1H), 6.76 (dd, $J = 2.3$ Hz, $J = 8.6$ Hz, 1H), 6.94 (d, $J = 2.3$ Hz, 1H). Ester **4j** was then reacted with hydrazine according to procedure A, to give **2j** as a yellow powder (72%). Purity 100%; ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 3.49 (dd, $J = 6.3$ Hz, $J = 11.7$ Hz, 1H), 3.63 (dd, $J = 3.0$ Hz, $J = 11.4$ Hz, 1H), 3.89 (br s, NH_2), 4.74 (dd, $J = 3.0$ Hz, $J = 6.3$ Hz, 1H), 6.55 (d, $J = 8.6$ Hz, 1H), 6.78 (dd, $J = 2.3$ Hz, $J = 8.6$ Hz, 1H), 6.87 (d, $J = 2.3$ Hz, 1H), 7.76 (br s, NH), MS (ESI+) *m*/*z* 228 $[M + H]^+$.

*4.3.14. 4-Biphenylacetic acid hydrazide (**2k**)*

A solution of 4-biphenylacetic acid (1 eq) and thionyl chloride (10 eq) in dry CH_2Cl_2 (10 mL) was stirred for 1 h at room temperature. The reaction mixture was quenched with EtOH and evaporated under reduced pressure to allow quantitative isolation of ethyl ester **4k**. The ester was then reacted with hydrazine according to procedure A, to give **2k** as a white powder. Yield 96%; Purity 99%; ^{1}H NMR (300 MHz, CD_3OD): δ (ppm): 3.63 (s, 2H), 3.90 (br s, NH_2), 6.69 (br s, NH), 7.34 (d, $J = 8.1$ Hz, 2H), 7.37 (t, $J = 7.5$ Hz, 1H), 7.46 (t, $J = 7.5$ Hz, 2H), 7.60 (dd, $J = 1.5$ Hz, $J = 8.1$ Hz, 4H), MS (ESI+) *m*/*z* 167 $[M + H]^+$.

*4.3.15. 2-(4-Chlorophenoxy)-2-methylpropanohydrazide (**2l**)*

2-(4-Chlorophenoxy)-2-methylpropanohydrazide (**2l**) was synthesized from the corresponding commercial ester and hydrazine according to procedure A as a white powder. Yield 98%; Purity 96%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.50 (s, 6H), 3.35 (br s, NH_2), 6.85 (d, $J = 9$ Hz, 2H), 7.25 (d, $J = 9$ Hz, 2H), 7.91 (br s, NH), MS (ESI+) *m*/*z* 229 $[M + H]^+$.

*4.3.16. Phenoxyacetic acid hydrazide (**2m**)*

Phenoxyacetic acid hydrazide (**2m**) was synthesized from the corresponding commercial ester and hydrazine according to procedure A as a white powder. Yield 98%; Purity 96%; ^{1}H NMR (300 MHz, CD_3OD): δ (ppm): 4.56 (s, 2H), 6.96–7.01 (m, 3H), 7.30 (dd, $J = 7.5$ Hz, $J = 8.1$ Hz, 2H), MS (ESI+) *m*/*z* 167 $[M + H]^+$.

*4.3.17. 4-Chloro-phenoxyacetic acid methylhydrazide (**2o**)*

To a 1.5 M solution of methylhydrazine in CH_2Cl_2 was added 4-chloro-phenoxyacetyl chloride (0.15 M in CH_2Cl_2) at 0 °C under argon. After stirring 1 h, the mixture was warmed to room temperature and stirred for 2 h. The reaction mixture was then poured into saturated Na_2CO_3 aqueous solution and extracted with CH_2Cl_2. The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to give the pure hydrazide **2o** as a white powder. Yield 97%; Purity 95%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 3.00 (s, 3H), 4.76 (s, NH_2), 4.93 (s, 2H), 5.04 (s, 2H), 6.85 (d, $J = 9.0$ Hz, 2H), 7.30 (d, $J = 9.0$ Hz, 2H), MS (ESI+) *m*/*z* 215 $[M + H]^+$.

*4.3.18. 1-(2-Amino-ethyl)-1H-pyridin-2-one (**3a**)*

To a stirred solution of 2-hydroxypyridine (4.5 mmol) in DMF (15 mL) was added NaH (60% mixture in mineral oil, 1.5 eq). The mixture was stirred at 80 °C under argon for 2 h, and then *N*-Boc-3-bromo-ethyl-amine (1 eq) was added. The mixture was stirred at 80 °C for 10 h, cooled to room temperature, quenched with water, and extracted with EtOAc. The combined organic layers were washed with brine, dried, and concentrated. The residue was washed with Et_2O to give white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.44 (s, 9H), 3.48 (dt, $J = 6.0$ Hz; 2H), 4.1 (t, $J = 6.0$ Hz; 2H), 4.99 (br, 1H), 6.18 (dd, $J = 3.0$ Hz, $J = 6.0$ Hz, 1H), 6.58 (d, $J = 9.0$ Hz, 1H), 7.25 (d, $J = 9.0$ Hz, 1H), 7.35 (dd, $J = 6.0$ Hz, $J = 3.0$ Hz, 1H). The protected amine was dissolved in 1 mL of a mixture of TFA/CH_2Cl_2 (v/v), the resulting mixture was stirred for 3 h at room temperature. The TFA salt was used directly for the library synthesis. ^{1}H NMR (300 MHz, CD_3OD): δ (ppm): 3.34 (t, $J = 5.7$ Hz, 2H) 4.29

(t, J = 5.7 Hz, 2H), 6.45 (dt, J = 1.2 Hz, J = 6.9 Hz, 1H), 6.60 (d, J = 9.0 Hz, 1H), 7.58 (ddd, J = 2.1 Hz, J = 6.9 Hz, J = 9.0 Hz, 1H), 7.63 (dd, J = 1.5 Hz, J = 6.9 Hz).

4.3.19. Methyl-1-aminoethylimidazole-5 or -4-carboxylate (**3b**)

To a stirred solution of methyl 4-imidazolecarboxylate (2 mmol) in DMF (10 mL) was added NaH (60% mixture in mineral oil, 1.5 eq). The mixture was stirred at 80 °C under argon for 2 h, and then *N*-Boc-3-bromo-ethyl-amine (1 eq) was added. The mixture was stirred at 80 °C for 3 h, and then cooled to room temperature. The reaction mixture was filtrated on Celite. Solvent was removed under reduced pressure and the crude product was quenched with water, and extracted with EtOAc (×3). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated. The residue was washed with Et_2O to give white solid. The protected amine was dissolved in 1 mL of 50/50 mixture of TFA/CH_2Cl_2, the resulting mixture was stirred for 3 h at room temperature, then concentrated under reduced pressure to give **3b** as a mixture of regioisomers. ^{1}H NMR (300 MHz, CD_3OD): δ (ppm): 3.28–3.32 (m, 4H), 3.76 (s, 3H), 3.82 (s, 2H), 4.31 (t, J = 6.3 Hz, 2H), 4.54 (t, J = 6.3 Hz, 2H), 7.78 (br s, 1H), 7.85 (d, J = 0.9 Hz, 1H), 8.01 (d, J = 1.3 Hz, 1H), 8.09 (br s, 1H), 8.20 (br s, NH^+ imidazole).

4.3.20. 4-(4-p-Fluorophenylpiperazin-1-yl)but-2-yn-1-amine (**3c**)

In a round-bottom flask, propargylamine (150.8 mmol, 10 mL, 1 eq) and methanol (60 mL) were chilled to 0 °C. Then Boc_2O (150.8 mmol, 1 eq) was added and the reaction mixture was allowed to stir 1 h at room temperature. At the end of the reaction (TLC), solvent was removed under reduced pressure and crude product was partitioned between water and CH_2Cl_2 (3 × 30 mL). The combined organic layers were joined, dried over $MgSO_4$ and evaporated under reduced pressure to give the *N*-Boc propargylamine as an orange solid. *N*-Boc propargylamine (3 g, 19.3 mmol, 1 eq), *N*-(4-fluorophenyl)piperazine (21.3 mmol, 1.1 eq), paraformaldehyde (23.2 mmol, 1.2 eq), acetic acid (3 mL) and dioxane (30 mL) and a spatula of CuCl were stirred at 70 °C for 3 h. After complete reaction of the piperazine, solvents were evaporated under reduced pressure and the residue was partitioned between a concentrated aq. NH_3 solution (pH 10) and CH_2Cl_2. The combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure. The crude product was then deprotected using a HCl/dioxane solution (3 N) to give **3c** as the dichlorhydrate salt. White powder. Yield 45%; ^{1}H NMR (300 MHz, CD_3OD) δ ppm 3.53–3.73 (br m, 8H piperazine); 3.99 (s, 2H); 4.33 (t, J = 3 Hz, 2H); 7.09 (t, J = 8.4 Hz, 2H), 7.24 (dd, J = 8.5 Hz, J = 3.0 Hz, 1H).

4.3.21. General procedure for acylthiosemicarbazide **1**, **10**–**48**, **53** synthesis (procedure D)

To a solution of 0.25 M di-pyridyl-thiocarbonate in DMF (stored on molecular sieves 3A) (1.05 eq) was added 0.1 M amine (free base) 0.1 M in DMF (1 eq). The reaction mixture was heated at 55 °C for 1.5 h. Then a solution of hydrazide (free base) 0.1 M in DMF (1 eq) was added. The reaction mixture was heated at 55 °C for another 1.5 h. Solvent was removed under reduced pressure. The residue was dissolved in EtOAc and the organic layer washed with water (3 × 20 mL) to remove pyridone, dried over $MgSO_4$ and concentrated under reduced pressure to give the desired compound. When needed, the compound was purified by preparative HPLC.

4.3.22. 1-(4-Chlorophenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**1**)

Synthesized following procedure D. White powder. Yield 65%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 4.60 (s, 2H), 4.78 (d, J = 5.7 Hz, 2H), 7.02 (dt, J = 9.0 Hz, 2H), 7.31–7.48 (m, 7H), 7.59–7.67 (m, 4H), 8.60 (br s, 1H NH), 9.44 (s, 1H NH), 10.17 (s, 1H NH). $t_{R,LCMS}$ = 3.45 min (10 min gradient), Purity 100%; MS (ESI+) m/z = 426 $[M + H]^+$.

4.3.23. 1-(4-Fluoropheonoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**10**)

White powder. Yield 100%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 4.58 (s, 2H), 4.78 (d, J = 6.0 Hz, 2H), 6.95–7.67 (m, 13H Ar), 8.6 (br s, NH), 9.44 (s, 1H, NH), 10.16 (s, 1H, NH). LC (method A) $t_{R,LCMS}$ = 3.45 min, Purity 100%; MS (ESI+) m/z = 410 $[M + H]^+$.

4.3.24. 1-(Phenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**11**)

Synthesized following procedure D. White powder. Yield 58%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.59 (s, 2H), 4.79 (d, J = 5.8 Hz, 2H), 6.97–7.00 (m, 3H), 7.27–7.49 (m, 7H), 7.59–7.67 (m, 4H), 8.61 (br s, 1H), 9.46 (s, 1H), 10.17 (s, 1H). $t_{R,LCMS}$ = 2.87 min, Purity 98%; MS (ESI+) m/z = 392 $[M + H]^+$.

4.3.25. 4-(4-Phenylbenzyl)-1-(4-trifluoromethylphenoxyacetyl)-thiosemicarbazide (**12**)

Synthesized following procedure D. White powder. Yield 32%; ^{1}H NMR (300 MHz, CD_3CN): δ (ppm): 4.72 (s, 1H), 4.85 (d, J = 6.1 Hz, 2H), 7.17 (d, J = 8.6 Hz, 2H), 7.35–7.49 (m, 6H), 7.60–7.68 (m, 8H). LC (method C) $t_{R,LCMS}$ = 3.06 min, Purity 94%; MS (ESI+) m/z = 460 $[M + H]^+$.

4.3.26. 1-(2-Chlorophenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**13**)

Synthesized following procedure D. White powder. Yield 53%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.74 (s, 2H), 4.81 (d, J = 5.5 Hz, 4H), 6.98 (dt, J = 1.1 Hz, J = 7.6 Hz, 1H), 7.09 (d, J = 8.0 Hz, 1H), 7.26 (dt, J = 1.5 Hz, J = 7.8 Hz, 1H), 7.36–7.49 (m, 6H), 7.59–7.67 (m, 4H), 8.65 (br s, 1H), 9.48 (s, 1H), 10.12 (s, 1H). $t_{R,LCMS}$ = 2.95 min, Purity 99%; MS (ESI+) m/z = 426 $[M + H]^+$.

4.3.27. 1-(3-Chlorophenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**14**)

Synthesized following procedure D. White powder. Yield 39%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.64 (s, 2H), 4.79 (d, J = 5.8 Hz, 2H), 6.96–7.04 (m, 2H), 7.09 (t, J = 2.1 Hz, 1H), 7.32 (d, J = 8.2 Hz, 1H), 7.35–7.48 (m, 5H), 7.59–7.67 (m, 4H), 8.63 (br s, 1H), 9.47 (s, 1H), 10.18 (s, 1H). $t_{R,LCMS}$ = 2.98 min, Purity 100%; MS (ESI+) m/z = 426 $[M + H]^+$.

4.3.28. 1-(2,4-Dichlorophenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**15**)

Synthesized following procedure D. White powder. Yield 34%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.78–4.81 (m, 4H), 7.12 (d, J = 8.9 Hz, 1H), 7.30–7.48 (m, 6H), 7.60–7.67 (m, 5H), 8.65 (br s, 1H), 9.47 (s, 1H), 10.14 (s, 1H). $t_{R,LCMS}$ = 3.10 min, Purity 95%; MS (ESI+) m/z = 460 $[M + H]^+$.

4.3.29. 1-(3,4-Dichlorophenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**16**)

Synthesized following procedure D. White powder. Yield 42%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.68–4.79 (m, 4H), 7.05 (d, J = 6.5 Hz, 1H), 7.32–7.64 (m, 12H), 8.65 (br s, 1H), 9.48 (s, 1H), 10.19 (s, 1H). $t_{R,LCMS}$ = 3.10 min, Purity 93%; MS (ESI+) m/z = 460 $[M + H]^+$.

4.3.30. 1-(4-Methoxyphenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**17**)

Synthesized following procedure D. White powder. Yield 47%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 3.71 (s, 3H), 4.41 (s, 2H), 4.83 (d, J = 5.0 Hz, 2H), 6.71 (d, J = 9.1 Hz, 2H), 6.79 (d, J = 9.1 Hz, 2H),

7.12 (br s, 1H), 7.36–7.47 (m, 6H), 7.53–7.61 (m, 5H). $t_{R,LCMS}$ = 2.82 min, Purity 92%; MS (ESI+) m/z = 422 $[M + H]^+$.

4.3.31. 1-(4-Benzyloxyphenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**18**)

Synthesized following procedure D. White powder. Yield 65%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 4.51 (s, 2H), 4.76 (d, J = 5.7 Hz, 2H), 5.01 (s, 2H), 6.92 (s, 4H), 7.31–7.48 (m, 10H), 7.59 (d, J = 7.7 Hz, 2H), 7.64 (d, J = 8.0 Hz, 2H), 8.55 (br s, 1H NH), 9.41 (s, 1H NH), 10.11 (s, 1H NH). $t_{R,LCMS}$ = 3.73 min. Purity 100%; MS (ESI+) m/z = 498 $[M + H]^+$.

4.3.32. 4-(4-Phenylbenzyl)-1-(4-phenylphenoxyacetyl)-thiosemicarbazide (**19**)

Synthesized following procedure D. White powder. Yield 35%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.66 (s, 2H), 4.80 (d, J = 5.6 Hz, 2H), 7.10 (d, J = 8.8 Hz, 2H), 7.31–7.50 (m, 8H), 7.59–7.66 (m, 8H), 8.63 (br s, 1H), 9.48 (s, 1H), 10.20 (s, 1H). $t_{R,LCMS}$ = 3.15 min, Purity 99%; MS (ESI+) m/z = 468 $[M + H]^+$.

4.3.33. 1-((2-Naphthyloxy)acetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**20**)

Synthesized following procedure D. White powder. Yield 38%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.73 (s, 2H), 4.80 (d, J = 5.8 Hz, 2H), 7.33–7.48 (m, 9H), 7.58–7.66 (m, 4H), 7.77–7.87 (m, 3H), 8.66 (br s, 1H), 9.49 (s, 1H), 10.26 (s, 1H). $t_{R,LCMS}$ = 3.05 min, Purity 98%; MS (ESI+) m/z = 442 $[M + H]^+$.

4.3.34. 1-((1-Naphthyloxy)acetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**21**)

Synthesized following procedure D. White powder. Yield 47%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.81 (m, 4H), 6.98 (d, J = 7.5 Hz, 1H), 7.35–7.55 (m, 9H), 7.59–7.67 (m, 4H), 7.87–7.90 (m, 1H), 8.44–8.47 (m, 1H), 8.68 (br s, 1H), 9.52 (s, 1H), 10.25 (s, 1H). $t_{R,LCMS}$ = 3.07 min, Purity 97%; MS (ESI+) m/z = 442 $[M + H]^+$.

4.3.35. 1-(4-Nitrophenoxyacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**22**)

Synthesized following procedure D. White powder. Yield 41%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.79 (m, 4H), 7.23 (d, J = 9.2 Hz, 2H), 7.36–7.48 (m, 5H), 7.59–7.66 (m, 4H), 7.28 (d, J = 9.2 Hz, 2H), 8.66 (br s, 1H), 9.49 (s, 1H), 10.28 (s, 1H). $t_{R,LCMS}$ = 2.83 min, Purity 95%; MS (ESI+) m/z = 437 $[M + H]^+$.

4.3.36. 1-(4-Chlorophenoxy-2-dimethylacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**23**)

Synthesized following procedure D. White powder. Yield 40%; ^{1}H NMR (300 MHz, CD_3CN): δ (ppm): 1.53 (s, 6H), 4.86 (d, J = 6.1 Hz, 2H), 7.00 (d, J = 8.9 Hz, 2H), 7.19 (br s, 1H), 7.27 (d, J = 8.9 Hz, 2H), 7.36–7.39 (m, 3H), 7.44–7.49 (m, 2H), 7.61–7.67 (m, 4H), 7.79 (br s, 1H), 8.95 (br s, 1H). $t_{R,LCMS}$ = 3.20 min, Purity 95%; MS (ESI+) m/z = 454 $[M + H]^+$.

4.3.37. 1-(6-Chloro-3,4-dihydro-2H-1,4-benzoxazine-2-carboxyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**24**)

Synthesized following procedure D. White powder. Yield 25%; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 3.35 (m, 2H), 4.70–4.77 (m, 3H), 6.06 (s, NH), 6.56 (d, J = 8.6 Hz, 1H), 6.71 (dd, J = 2.3 Hz, J = 8.6 Hz, 1H), 6.80 (d, J = 2.3 Hz, 1H), 7.38 (m, 2H), 7.46 (t, J = 7.8 Hz, 2H), 7.58–7.67 (m, 4H), 8.43 (br s, 1H, NH), 9.44 (s, 1H, NH), 10.14 (s, 1H, NH). $t_{R,LCMS}$ = 6.62 min (10 min gradient), Purity 96%; MS (ESI+) m/z 453 $[M + H]^+$.

4.3.38. 1-(4-Chlorobenzoyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**25**)

Synthesized following procedure D. White powder. Yield 65%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 4.78 (d, J = 5.6 Hz, 2H), 7.35–7.48 (m, 5H), 7.56–7.67 (m, 6H), 7.96 (dt, J = 8.6 Hz, 2H), 8.74 (br s, 1H NH), 9.52 (s, 1H NH), 10.53 (s, 1H NH). $t_{R,LCMS}$ = 3.60 min, Purity 100%; MS (ESI+) m/z = 396 $[M + H]^+$.

4.3.39. 1-(4-Chlorophenylaminoacetyl)-4-(4-phenylbenzyl)-thiosemicarbazide (**26**)

Synthesized following procedure D. White powder. Yield 21%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 3.78 (d, J = 5.8 Hz, 2H), 4.79 (d, J = 5.8 Hz, 2H), 6.07 (t, J = 5.8 Hz, 1H), 6.62 (d, J = 8.8 Hz, 2H), 7.10 (d, J = 8.8 Hz, 2H), 7.33–7.38 (m, 3H), 7.44–7.49 (m, 2H), 7.59–7.67 (m, 4H), 8.48 (br s, 1H), 9.43 (s, 1H), 10.00 (s, 1H). $t_{R,LCMS}$ = 2.93 min, Purity 96%; MS (ESI+) m/z = 425 $[M + H]^+$.

4.3.40. 1-(4-Chlorophenoxyacetyl)-4-(3-phenylbenzyl)-thiosemicarbazide (**27**)

Synthesized following procedure D. White powder. Yield 57%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 4.03 (s, 2H) 4.60 (s, 2H), 7.01 (d, J = 9.0 Hz, 2H), 7.25–7.55 (m, 9H), 7.61–7.67 (m, 3H), 8.65 (br s, 1H NH), 9.43 (s, 1H NH), 10.17 (s, 1H NH). $t_{R,LCMS}$ = 3.45 min (10 min gradient), Purity 100%; MS (ESI+) m/z = 426 $[M + H]^+$.

4.3.41. 1-(4-Chlorophenoxyacetyl)-4-(2-phenylbenzyl)-thiosemicarbazide (**28**)

Synthesized following procedure D. White powder. Yield 81%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.57 (s, 2H), 4.64 (d, J = 5.7 Hz, 2H), 7.01 (m, J = 8.7 Hz, 2H), 7.21 (m, 1H), 7.28–7.50 (m, 10H), 8.44 (br s, 1H, NH), 9.44 (s, 1H, NH), 10.16 (s, 1H, NH). $t_{R,LCMS}$ = 3.46 min (10 min gradient), Purity 98.5%; MS (ESI+) m/z = 426 $[M + H]^+$.

4.3.42. 4-Benzyl-1-(4-chlorophenoxyacetyl)-thiosemicarbazide (**29**)

Synthesized following procedure D. White powder. Yield 83%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.57 (s, 2H), 4.64 (d, J = 5.7 Hz, 2H), 7.05 (d, J = 8.7 Hz, 2H), 7.28–7.50 (m, 7H), 8.54 (br s, 1H, NH), 9.45 (s, 1H, NH), 10.15 (s, 1H, NH). $t_{R,LCMS}$ = 5.55 min (10 min gradient), Purity 97.5%; MS (ESI+) m/z = 350 $[M + H]^+$.

4.3.43. 2-(4-Chlorophenoxy)-N-([(pyridin-3-ylmethyl) carbamothioyl]amino)acetamide (**30**)

Synthesized following procedure D. White powder. Yield 63%; ^{1}H NMR 300 MHz (DMSO-d_6): δ (ppm) 4.59 (s, 2H), 4.75 (d, J = 5.7 Hz, 2H), 7.01 (d, J = 9.0 Hz, 2H), 7.32–7.34 (m, 3H), 7.68 (d, J = 7.8 Hz, 1H), 8.42 (d, J = 3.6 Hz, 1H), 8.50 (s, 1H), 8.61 (br s, 1H NH), 9.49 (br s, 1H NH), 10.17 (br s, 1H NH). ^{13}C NMR 75 MHz (DMSO-d_6): δ (ppm) 44.8, 66.8, 117.0, 123.7, 125.4, 129.7, 135.2, 148.4, 149.0, 157.0, 167.7. $t_{R,LCMS}$ = 2.52 min (5 min gradient), Purity 97%; MS (ESI+) m/z = 351 $[M + H]^+$. HRMS m/z calculated for $C_{15}H_{14}ClN_4OS$ $[M - H_2O + H]^+$ 333.0577, found 333.0616.

4.3.44. 1-(4-Chlorophenoxyacethyl)-4-phenethyl-thiosemicarbazide (**31**)

Synthesized following procedure D. White powder. Yield 88%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 2.80–2.87 (m, 2H), 3.55–3.62 (m, 2H), 4.61 (s, 2H), 7.06 (d, J = 8.6 Hz, 2H), 7.21–7.36 (m, 7H), 8.12 (br s, 1H, NH), 9.45 (s, 1H, NH), 10.08 (s, 1H, NH). $t_{R,LCMS}$ = 5.65 min (10 min gradient), Purity 99.5%, MS (ESI+) m/z = 364 $[M + H]^+$.

4.3.45. 1-(4-Chlorophenoxyacethyl)-4-(3-imidazol-1-yl-propyl)-thiosemicarbazide (**32**)

Synthesized following procedure D. White powder. Yield 64%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 1.94 (qt, J = 6.0 Hz, J = 6.0 Hz, 2H), 3.41 (q, J = 6.0 Hz, J = 12.0 Hz, 2H), 3.94 (t, J = 6.0 Hz, 2H), 4.59 (s, 2H), 6.88 (s, 1H), 7.03 (dt, J = 9.0 Hz, J = 2.0 Hz, 2H), 7.15

(s, 1H), 7.35 (dt, J = 9.0 Hz, J = 2.0 Hz, 2H), 7.61 (s, 1H), 8.08 (t, J = 12.0 Hz, 1H, NH), 9.32 (s, 1H, NH), 10.08 (s, 1H, NH). $t_{R,LCMS}$ = 2.72 min, Purity 98.8%; MS (ESI+) m/z = 368 $[M + H]^+$.

4.3.46. 1-(4-Chlorophenoxyacethyl)-4-phenylpropyl-thiosemicarbazide (**33**)

Synthesized following procedure D. White powder. Yield 88%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 1.74–1.84 (m, 2H), 2.50–2.57 (m, 2H), 3.42–3.48 (m, 2H), 4.61 (s, 2H), 7.02 (d, J = 8.6 Hz, 2H), 7.21–7.34 (m, 7H), 8.05 (br s, 1H, NH), 9.25 (s, 1H, NH), 10.05 (s, 1H, NH). $t_{R,LCMS}$ = 5.87 min (10 min gradient), Purity 98.5%, MS (ESI+) m/z = 378 $[M + H]^+$.

4.3.47. 1-(4-Chlorophenoxyacethyl)-4-phenylbutyl-thiosemicarbazide (**34**)

Synthesized following procedure D. White powder. Yield 92%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 1.45–1.52 (m, 4H), 2.55–2.59 (t, J = 6.9 Hz, 2H), 3.36 (s, 2H), 4.42–4.46 (m, 2H), 7.00 (d, J = 8.6 Hz, 2H), 7.15–7.34 (m, 7H), 8.00 (s, 1H, NH), 9.19 (s, 1H, NH), 10.01 (s, 1H, NH). $t_{R,LCMS}$ = 6.52 min (10 min gradient), Purity 99.5%; MS (ESI+) m/z = 392 $[M + H]^+$.

4.3.48. 1-(4-chlorophenoxyacethyl)-4-cyclohexylbutyl-thiosemicarbazide (**35**)

Synthesized following procedure D. White powder. Yield 78%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 0.81–0.88 (m, 2H), 1.10–1.25 (m, 8H), 1.37–1.48 (m, 2H), 1.55–1.69 (m, 5H), 3.37–3.47 (m, 2H), 4.57 (s, 2H), 7.01 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 8.7 Hz, 2H), 7.92 (br s, 1H, NH), 9.17 (s, 1H, NH), 10.00 (s, 1H, NH). ^{13}C NMR 75 MHz (DMSO): δ (ppm): 24.0, 26.3, 26.7, 29.5, 33.4, 37.1, 37.5, 44.2; 67.7, 116.9, 125.3; 126.7, 157.1, 167.6. $t_{R,LCMS}$ = 3.62 min, Purity 99.5%, MS (ESI+) m/z = 398 $[M + H]^+$.

4.3.49. 4-Cyclohexylbutyl-1-(4-trifluoromethylphenoxyacethyl)-thiosemicarbazide (**36**)

Synthesized following procedure D. White powder. Yield 68%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 0.71–0.88 (m, 2H), 1.08–1.31 (m, 8H), 1.37–1.51 (m, 2H), 1.55–1.69 (m, 5H), 3.35–3.45 (m, 2H), 4.68 (s, 2H), 7.17 (d, J = 8.7 Hz, 2H), 7.65 (d, J = 8.7 Hz, 2H), 7.95 (br s, 1H, NH), 9.17 (s, 1H, NH), 10.05 (s, 1H, NH). $t_{R,LCMS}$ = 3.82 min, Purity 99.5%; MS (ESI+) m/z = 432 $[M + H]^+$.

4.3.50. N-([(4-Cyclohexylbutyl)carbamothioyl]amino)-2-phenoxyacetamide (**37**)

Synthesized following procedure D. White powder. Yield 52%; ^{1}H NMR 300 MHz (DMSO-d_6): δ (ppm) 0.83–0.92 (m, 2H), 1.24–1.45 (m, 8H), 1.40–1.50 (m, 2H), 1.61–1.68 (m, 5H), 4.36–4.40 (m, 2H), 4.56 (s, 2H), 6.98 (d, 3H, J = 7.8 Hz), 7.30 (t, 2H, J = 8.7 Hz), 7.91 (br s, 1H), 9.18 (br s, 1H), 10.0 (br s, 1H). ^{13}C NMR 75 MHz (DMSO-d_6): δ (ppm) 24.0, 26.3, 26.7, 29.5, 33.3, 37.2, 37.4, 44.2, 66.5, 115.1, 121.6, 129.9, 158.2, 167.8, 181.8. $t_{R,LCMS}$ = 3.48 min, (5 min gradient), Purity 95%; MS (ESI+) m/z = 364 $[M + H]^+$. HRMS m/z calculated for $C_{19}H_{28}N_3OS$ $[M - H_2O + H]^+$ 346.1953, found 346.1949.

4.3.51. N-([(4-Cyclohexylbutyl)carbamothioyl]amino)-2-(4-methoxyphenoxy)acetamide (**38**)

Synthesized following procedure D. White powder. Yield 56%; ^{1}H NMR 300 MHz (DMSO-d_6): δ (ppm) 0.75–1.07 (m, 2H), 1.16–1.24 (m, 8H), 1.39–1.49 (m, 2H), 1.61–1.68 (m, 5H), 3.36–3.42 (m, 2H), 3.69 (s, 3H), 4.49 (s, 2H), 6.81–6.94 (m, 4H), 7.87 (br s, 1H), 9.16 (br s, 1H), 9.95 (br s, 1H). ^{13}C NMR 75 MHz (DMSO-d_6): δ (ppm) 24.0, 26.3, 26.7, 29.5, 33.3, 37.2, 37.4, 44.2, 55.8, 67.2, 115.0, 116.1, 152.2, 154.2, 168.0, 182.1. $t_{R,LCMS}$ = 3.43 min, (5 min gradient), Purity 100%; MS (ESI+) m/z = 394 $[M + H]^+$. HRMS m/z calculated for $C_{20}H_{32}N_3O_3S$ $[M + H]^+$ 394.2164, found 394.2177.

4.3.52. 4-Cyclohexylbutyl-1-(4-nitrophenoxyacethyl)-thiosemicarbazide (**39**)

Synthesized following procedure D. White powder. Yield 68%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 0.75–0.88 (m, 2H), 1.05–1.30 (m, 8H), 1.39–1.49 (m, 2H), 1.55–1.69 (m, 5H), 3.36–3.46 (m, 2H), 4.77 (s, 2H), 7.20 (d, J = 8.7 Hz, 2H), 7.99 (br s, 1H, NH), 8.21 (d, J = 8.7 Hz, 2H), 9.20 (s, 1H, NH), 10.10 (s, 1H, NH). $t_{R,LCMS}$ = 3.77 min, Purity 99.5%; MS (ESI+) m/z = 409 $[M + H]^+$.

4.3.53. 4-Cyclohexylbutyl-1-(1-naphthyloxyacethyl)-thiosemicarbazide (**40**)

Synthesized following procedure D. White powder. Yield 68%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 0.72–0.92 (m, 2H), 1.06–1.30 (m, 8H), 1.40–1.48 (m, 2H), 1.55–1.69 (m, 5H), 3.37–3.47 (m, 2H), 4.77 (s, 2H), 6.95 (d, J = 7.7 Hz, 1H), 7.41 (dd, J = 7.7 Hz, J = 7.7 Hz, 1H), 7.47–7.57 (m, 3H), 7.78 (d, J = 7.7 Hz, 1H), 7.96 (br s, 1H, NH), 8.43 (d, J = 7.7 Hz, 1H), 9.25 (s, 1H, NH), 10.08 (s, 1H, NH). $t_{R,LCMS}$ = 3.95 min, Purity 99.5%; MS (ESI+) m/z = 409 $[M + H]^+$.

4.3.54. 1-(6-Chloro-3,4-dihydro-2H-benzo [1,4]oxazin-2-carbonyl)-4-(cyclohexylbutyl)-thiosemicarbazide (**41**)

Synthesized following procedure D. White powder. Yield 16%; ^{1}H NMR (300 MHz, CD_3CN): δ (ppm): 0.87–0.94 (m, 2H), 1.11–1.20 (m, 8H), 1.41–1.52 (m, 2H), 1.69–1.73 (m, 5H), 3.45–3.51 (m, 4H), 4.75 (br s, 1H), 4.87 (t, J = 8.3 Hz, 1H), 6.69 (d, J = 8.5 Hz, 1H), 6.82 (dd, J = 2.2 Hz, J = 8.4 Hz, 2H), 6.93 (d, J = 2.2 Hz, 1H), 7.66 (s, 1H), 8.74 (s, 1H). $t_{R,LCMS}$ = 2.95 min, Purity 95%; MS (ESI+) m/z = 426 $[M + H]^+$.

4.3.55. 1-(4-Biphenylacethyl)-4-cyclohexylbutyl-thiosemicarbazide (**42**)

Synthesized following procedure D. White powder; yield 79%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 0.75–0.93 (m, 2H), 1.13–1.23 (m, 8H), 1.42–1.47 (m, 2H), 1.62–1.67 (m, 5H), 3.41 (m, 2H), 3.52 (s, 2H), 7.34–7.47 (m, 5H), 7.58–7.67 (m, 4H), 7.84 (br s, 1H, NH), 9.15 (s, 1H, NH), 9.93 (s, 1H, NH). t_R = 6.75 min (10 min gradient), Purity 99.5%; MS (ESI+) m/z = 424 $[M + H]^+$.

4.3.56. 4-Cyclohexylbutyl-1-(phenyl-[1,2,4]oxadiazol-5-yl-acetyl)-thiosemicarbazide (**43**)

Synthesized following procedure D. White powder. Yield 68%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 0.73–0.91 (m, 2H), 1.06–1.33 (m, 7H), 1.49–1.72 (m, 9H), 3.52–3.60 (m, 2H), 4.09 (s, 2H), 6.95 (br s, 1H NH), 7.50–7.55 (m, 3H), 8.03–8.09 (m, 2H), 8.69 (s, 1H NH), 9.89 (s, 1H NH). $t_{R,LCMS}$ = 5.97 min (10 min gradient), Purity 99% MS (ESI+) m/z = 416 $[M + H]^+$.

4.3.57. 4-(Cyclohexylbutyl)-1-(3-pyrazin-2-yl-[1,2,4]oxadiazol-5-yl-acetyl)-thiosemicarbazide (**44**)

Synthesized following procedure D. White powder. Yield 50%; ^{1}H NMR (DMSO-d_6): δ (ppm): 0.75–0.83 (m, 2H), 1.11–1.23 (m, 8H), 1.60–1.66 (m, 7H), 3.45–3.50 (m, 2H), 4.16 (s, 2H), 8.03 (br s, NH), 8.88 (d, J = 2.4 Hz, 2H), 9.26 (s, 1H), $t_{R,LCMS}$ = 3.46 min, MS (ESI+) m/z = 418 $[M + H]^+$.

4.3.58. N-([(4-Cyclohexylbutyl)carbamothioyl]amino)acetamide (**45**)

Synthesized following procedure D. White powder. Yield 17%; ^{1}H NMR 300 MHz (DMSO-d_6): δ (ppm) 0.76–0.89 (m, 2H), 1.07–1.20 (m, 8H), 1.39–1.46 (m, 2H), 1.63–1.68 (m, 5H), 1.64 (s, 3H), 3.37–3.39 (m, 2H), 7.86 (br s, 1H), 8.99 (br s, 1H), 9.59 (br s, 1H). ^{13}C NMR 75 MHz (DMSO-d_6): δ (ppm) 21.4, 24.0, 26.3, 26.7, 29.5, 33.3, 37.1, 37.5, 44.1, 169.4, 181.8. $t_{R,LCMS}$ = 3.13 min (5 min gradient), Purity 93%; MS (ESI+) m/z = 272 $[M + H]^+$. HRMS m/z calculated for $C_{13}H_{26}N_3OS$ $[M + H]^+$ 272.1797, found 272.1805.

4.3.59. 4-Chloro-N-([(4-cyclohexylbutyl)carbamothioyl]amino) benzamide (**46**)

Synthesized following procedure D. White powder. Yield 90%; ^{1}H NMR 300 MHz (DMSO-d_6): δ (ppm) 0.80–0.84 (m, 2H), 1.15–1.29 (m, 8H), 1.43–1.45 (m, 2H), 1.62–1.66 (m, 5H), 3.38–3.44 (m, 2H), 7.57 (d, J = 8.4 Hz, 2H), 7.9 (d, J = 8.4 Hz, 2H), 8.08 (br s, 1H), 9.22 (br s, 1H), 10.37 (br s, 1H). ^{13}C NMR 75 MHz (DMSO-d_6): δ (ppm) 24.0, 26.3, 26.7, 29.5, 33.3, 37.1, 37.4, 44.2, 128.8, 130.2, 131.8, 137.1, 165.4, 182.0. $t_{R,LCMS}$ = 3.52 min, (5 min gradient), Purity 100%; MS (ESI+) m/z = 368 $[M + H]^+$. HRMS m/z calculated for $C_{18}H_{27}N_3OSCl$ $[M + H]^+$ 368.1563, found 368.1588.

4.3.60. 1-(4-Chlorophenoxyacetyl)-1-methyl-4-(4-phenylbenzyl)-thiosemicarbazide (**47**)

Synthesized following procedure D. White powder. Yield 11%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 3.00 (s, 3H), 4.51 (m, 1H), 4.74–4.94 (m, 3H), 6.88 (d, J = 7.6 Hz, 2H), 7.28 (d, J = 8.9 Hz, 2H), 7.34–7.47 (m, 5H), 7.58–7.62 (m, 4H), 9.23 (br s, 1H), 9.74 (s, 1H). $t_{R,LCMS}$ = 3.12 min, Purity 99%; MS (ESI+) m/z = 440 $[M + H]^+$.

4.3.61. 2-(4-Chlorophenoxy)-N-([(4-cyclohexylbutyl) carbamothioyl]amino)-N-methylacetamide (**48**)

Synthesized following procedure D. White powder. Yield 15%; ^{1}H NMR 300 MHz (DMSO-d_6): δ (ppm) 0.82–0.91 (m, 2H), 1.15–1.29 (m, 10H), 1.48–1.64 (m, 7H), 2.95 (s, 3H), 3.44–3.51 (m, 2H), 4.86 (m, 1H), 6.90 (d, 2H, J = 9.0 Hz), 7.29 (d, 2H, J = 9.0 Hz), 8.59 (br s, 0.3H), 9.45 (br s, 0.7H). $t_{R,LCMS}$ = 3.72 min, (5 min gradient), Purity 97%; MS (ESI+) m/z = 412 $[M + H]^+$. HRMS m/z calculated for $C_{20}H_{29}ClN_3OS$ $[M - H_2O + H]^+$ 394.1720, found 394.1726.

4.3.62. 2-(4-Chlorophenoxy)-N-[(4-phenylphenyl)methylamino]-2-thioxo-ethyl]acetamide (**49**)

To a solution of *N*-Boc-glycine (2 mmol, 1 eq), **3ac** (1 eq), ECDI (1.1 eq), HOBt (1.1 eq) in 3 mL of DCM was added Et_3N (2.5 eq). The resulting mixture was stirred for 3 h at room temperature. After evaporation, the crude product was dissolved EtOAc and the organic layer was washed with aq. $NaHCO_3$ 5% (3×), 0.1 N aq. $KHSO_4$ (3×) and saturated aq. NaCl. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure to give *N*-(4phenylbenzyl)-2-(*tert*-butoxycarbonylamino)acetamide. Yield 45%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.45 (s, 9H), 3.86 (d, J = 5.7 Hz, 2H), 4.52 (d, J = 5.7 Hz, 2H), 5.13 (br s, NH), 6.44 (br s, NH), 7.32–7.38 (m, 3H), 7.45 (t, J = 7.2 Hz, 2H), 7.55–7.59 (m, 4H). The amide (0.88 mmol, 1 eq) was reacted with Lawesson reagent (1 eq) in THF (3 mL). The mixture was stirred 18 h at room temperature. After evaporation of solvent under reduced pressure, the crude product was purified by preparative HPLC to give the corresponding thioamide. Yield 67%; ^{1}H NMR ($CDCl_3$): δ (ppm): 1.43 (s, 9H), 4.25 (d, J = 5.1 Hz, 2H), 4.92 (d, J = 5.1 Hz, 2H), 5.32 (br s, NH), 7.38–7.41 (m, 3H), 7.47 (t, J = 7.2 Hz, 2H), 7.56–7.61 (m, 4H), 8.55 (br s, NH). Previous compound (0.55 mmol, 1 eq) was dissolved in 3 mL of a 50/50 mixture of CH_2Cl_2/TFA, and the resulting mixture was stirred for 3 h at room temperature. Solvents were removed under reduced pressure to give the free amine quantitatively. ^{1}H NMR (300 MHz, CD_3OD): δ (ppm): 3.90 (s, 2H), 4.92 (s, 2H), 7.34 (t, J = 7.2 Hz, 1H), 7.42–7.48 (m, 4H), 7.60–7.64 (m, 4H). The free amine (0.4 mmol, 1 eq) was dissolved in 4 mL of CH_2Cl_2. Et_3N (2.5 eq) and 4-chlorophenoxyacetyl chloride (1 eq) were added. The resulting mixture was stirred for 3 h at room temperature. After evaporation, the crude product was dissolved EtOAc and the organic layer was washed with aq. $NaHCO_3$ 5% (3×), 0.1 N aq. $KHSO_4$ (3×) and saturated aq. NaCl. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure to give (**49**) as an oil. Yield 35%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 4.20 (d, J = 5.9 Hz, 2H), 4.57 (s, 2H), 4.87 (d, J = 5.1 Hz, 2H), 7.00–7.04 (m, 2H), 7.32–7.39 (m, 5H), 7.45 (t, J = 6.9 Hz, 2H), 7.60–7.65 (m, 4H), 8.57 (t, J = 5.9 Hz, NH), 10.28 (br s, 1H, NH), $t_{R,LCMS}$ = 7.50 min (10 min gradient), Purity 95%; MS (ESI+) m/z 425 $[M + H]^+$.

4.3.63. 1-(4-Chlorophenoxyacetamido)-3-(4-phenylbenzyl)-1,3-dihydro-2H-imidazole-2-thione (**50**)

To a solution of **1** (0.235 mmol, 1 eq) in CH_2Cl_2 (5 mL) were added NEt_3 (32 μL, 1 eq) and chloroacetaldehyde 45% w/w in water (41 μL, 5 eq). The reaction mixture was stirred 18 h at room temperature. Then 40 μL of TFA were added and the reaction stirred for 15 min. The organic layer was washed with water (3 × 5 mL), dried over $MgSO_4$ and concentrated under reduced pressure to give **50** as an oil. Yield 71%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 4.68 (s, 2H), 5.25 (s, 2H), 6.50 (d, J = 6 Hz, 1H), 6.74 (d, J = 6 Hz, 1H), 6.89 (d, J = 9 Hz, 2H), 7.26–7.29 (m, 2H), 7.38–7.49 (m, 5H), 7.56–7.64 (m, 4H), 8.60 (br s, 1H), $t_{R,LCMS}$ = 6.25 min (10 min gradient), Purity 97%; m/z (ESI+) m/z 450 $[M + H]^+$.

4.3.64. 2-(4-Chlorophenoxy)-N-([methyl(4-phenylbutyl) carbamothioyl]amino)acetamide (**51**)

To a suspension of TCDI (230 mg, 1.29 mmol, 1.1 eq) in anhydrous THF (2.6 mL) was added the hydrazide **2q** (235 mg, 1.17 mmol, 1 eq) and the reaction mixture was refluxed for 24 h. The reaction mixture was cooled, the amine *N*-methyl-(4-phenylbutyl) amine (246 mg, 1.23 mmol, 1.05 eq) was added and the reaction mixture was stirred for a further 72 h. The crude reaction mixture was diluted with EtOAc and washed with 1 N HCl, H_2O, brine, dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography on silica gel (cyclohexane/EtOAc 1:0 to 1:1 (v/v)) to afford the desired compound (106 mg). White powder. Yield 22%; ^{1}H NMR 300 MHz (DMSO-d_6): δ (ppm) 1.52–1.59 (m, 4H), 2.59 (t, 2H, J = 5.7 Hz), 3.10 (s, 3H), 3.70–3.76 (m, 2H), 4.60 (s, 2H), 7.03 (d, 2H, J = 9.0 Hz), 7.16–7.34 (m, 8H), 9.28 (s, 1H, NH), 10.09 (s, 1H, NH). ^{13}C NMR 75 MHz (DMSO-d_6): δ (ppm) 26.7, 28.5, 35.4, 41.1, 66.7, 117.1, 125.3, 126.1, 128.7, 129.6, 142.5, 157.1, 167.1, 183.2. $t_{R,LCMS}$ = 3.33 min, (5 min gradient), Purity 97%; MS (ESI+) m/z = 406 $[M + H]^+$. HRMS m/z calculated for $C_{20}H_{25}ClN_3O_2S$ $[M + H]^+$ 406.1356, found 406.1345.

4.3.65. 4-Benzylpiperidine-1-(4-chlorophenoxyacethyl)-thiosemicarbazide (**52**)

Compound **52** was synthesized using the same protocol as **51** from hydrazide **2q** and 4-benzylpiperidine. White powder. Yield 74%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.21–1.39 (m, 2H), 1.75 (d, J = 13.5 Hz, 2H), 1.81–1.92 (m, 1H), 2.56 (d, J = 7.5 Hz, 2H), 3.01 (dt, J = 3 Hz, J = 13.5 Hz, 2H), 4.61 (s, 2H), 4.64–4.67 (m, 2H), 6.94 (d, J = 8.9 Hz, 2H), 7.12–7.36 (m, 7H), 8.86 (s, 1H, NH), 10.08 (s, 1H, NH). $t_{R,LCMS}$ = 5.17 min (10 min gradient), Purity 99%; MS (ESI+) m/z = 418 $[M + H]^+$.

4.3.66. 1-(4-Biphenylacetyl)-4-(4-chlorophenethyl)-thiosemicarbazide (**53**)

Synthesized following procedure D. White powder. Yield 77%; ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm): 2.78–2.82 (m, 2H), 3.51 (s, 2H), 3.59–3.63 (m, 2H), 7.21–7.26 (m, 2H), 7.35–7.48 (m, 7H), 7.57–7.66 (m, 4H), 8.05 (br s, 1H, NH), 9.23 (s, 1H, NH), 9.98 (s, 1H, NH). $t_{R,LCMS}$ = 6.75 min (10 min gradient), Purity 99%; MS (ESI+) m/z = 424 $[M + H]^+$.

4.3.67. 3-[2-(4-Chloro-phenoxy)-acetylamino]-2-mercapto-N-phenethyl-propionamide (**54**)

3-[2-(4-Chloro-phenoxy)-acetylamino]-2-mercapto-*N*-phenethyl-propionamide (**54**) was prepared in 3 steps from thiomalic acid. First, a round bottom flask containing conc. H_2SO_4 (5 mL) at room temperature and a receiver flask with thiomalic acid

(10 mmol) in anhydrous DMSO (4 mL) at room temperature were joined by a distillation unit. H_2SO_4 was stirred while hexafluoroacetone trihydrate (4.2 mL, 3.5 eq) was added dropwise with a dropping funnel over 1 h. A white gas was observed. The reaction mixture was vigorously stirred under the hexafluoroacetone atmosphere at room temperature for 12 h. The reaction mixture was diluted in H_2O (10 mL) and extracted (5×) with CH_2Cl_2. The organic layer was washed with sat. aq. NaCl, then compound was partitioned between CH_2Cl_2 and aq. $NaHCO_3$. The aqueous layer was acidified with HCl 1 N and extracted with CH_2Cl_2 (5×). The combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure to give [2,2-bis(trifluoromethyl)-5-oxo-1,3-oxathiolan-4-yl]acetic acid. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 3.00 (dd, $J = 10.2$ Hz, $J = 18$ Hz, 1H), 3.36 (dd, $J = 18$ Hz, $J = 3.3$ Hz, 1H), 4.51 (dd, $J = 3.3$ Hz, $J = 10.2$ Hz, 1H). ^{19}F NMR (282.4 MHz, $CFCl_3CDCl_3$): δ (ppm) −76.88 (q, $J = 9.6$ Hz, 3F), −76.99 (q, $J = 9.6$ Hz, 3F). A solution of former carboxylic acid (0.16 mmol) and $SOCl_2$ (5 eq) in CH_2Cl_2 (2 mL) was refluxed 6 h. The reaction was cooled and concentrated under reduced pressure. The crude acid chloride was dissolved in toluene (2 mL) in the presence of $TMSN_3$ (1 eq). The reaction was refluxed 18 h and then evaporated under reduced pressure to give the isocyanate that was directly reacted with 4-chlorophenoxyacetic acid (5 eq) in refluxed toluene (2 mL) during 18 h to give the mixed anhydride, which eliminates carbon dioxide to give the amide. The crude amide was purified using flash chromatography (cyclohexane/EtOAc). Then the 5-oxo-1,3-oxathiolane ring (0.02 mmol) was reacted with phenethylamine (1 eq) in Et_2O (2 mL) to give quantitatively **54**. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 1.88 (d, $J = 8.7$ Hz, 1H), 2.71 (t, $J = 7.1$ Hz, 2H), 3.33–3.52 (m, 4H), 3.60–3.66 (m, 2H), 4.00–4.07 (m, 1H), 4.37 (s, 2H), 6.31–6.33 (m, 1H), 6.76–6.79 (m, 2H), 7.01–7.25 (m, 14H), $t_{R,LCMS} = 3.54$ min, Purity 92%; MS (ESI+) m/z 392 $[M + H]^+$.

Acknowledgments

The authors would like to thank V. Pilato, E. Carasso, L. Gaquere and S. Delaroche for technical help. We are grateful to the institutions that support our laboratory (INSERM, School of Pharmacy Lille, Université Lille Nord de France, and Institut Pasteur de Lille). Data management was performed using Pipeline Pilot™ from Accelrys Inc and Miner3D™ softwares. This project was supported by the Fondation pour la Recherche Medicale; Nord-Pas-de-Calais (RAD07001EEA) and PRIM (Pôle de Recherche Interdisciplinaire pour le Médicament). We thank the LARMNS NMR laboratory, School of Pharmacy Lille.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.08.027.

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