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N-Aryl-*N'*-ethyleneaminothioureas Effectively Inhibit Acetylcholinesterase 1 from Disease-Transmitting Mosquitoes

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Abstract: Vector control of disease-transmitting mosquitoes by insecticides has a central role in reducing the number of parasitic- and viral infection cases. The currently used insecticides are efficient, but safety concerns and the development of insecticide-resistant mosquito strains warrant the search for alternative compound classes for vector control. Here, we have designed and synthesized thiourea-based compounds as non-covalent inhibitors of acetylcholinesterase 1 (AChE1) from the mosquitoes *Anopheles gambiae* (*An. gambiae*) and *Aedes aegypti* (*Ae. aegypti*), as well as a naturally occurring resistant-conferring mutant. The *N*-aryl-*N'*-ethyleneaminothioureas proved to be inhibitors of AChE1; the most efficient one showed submicromolar potency. Importantly, the inhibitors exhibited selectivity over the human AChE (*h*AChE), which is desirable for new insecticides. The structure-activity relationship (SAR) analysis of the thioureas revealed that small changes in the chemical structure had a large effect on inhibition capacity. The thioureas showed to have different SAR when inhibiting AChE1 and *h*AChE, respectively, enabling an investigation of structure-selectivity relationships. Furthermore, insecticidal activity was demonstrated using adult and larvae *An. gambiae* and *Ae. aegypti* mosquitoes.

1. Introduction

Mosquitoes of the *Anopheles* and *Aedes* genera act as vectors for parasitic and viral infectious diseases that affect the health of hundreds of millions of people every year. With 40% of the world's population at risk, dengue is now considered one of the fastest spreading vector-borne diseases and poses a serious public health threat [1-3]. National programs and international initiatives to increase the coverage of mosquito-control interventions have resulted in a decline in the incidence of the deadliest vector-borne disease, malaria [4]. Still, in 2016, nearly 1.1 billion people remained at high risk of malaria infection, with young children being among the most vulnerable [5]. Current mosquito-control interventions are primarily based on insecticide-treated bed nets (ITN) and indoor residual spraying (IRS). These methods rely on four classes of insecticides, and their effectiveness is seriously threatened by the development and spread of insecticide-resistant mosquito populations [5-7]. Accordingly, there is a need for new strategies and chemicals for vector control in order to maintain or accelerate the current progress of achieving large-scale reduction of vector-borne diseases [8-12].

The essential enzyme acetylcholinesterase (AChE, EC 3.1.1.7), is pivotal in cholinergic neurotransmission and a validated insecticidal target [13, 14]. The active site gorge of AChEs is lined with aromatic residues and has two partly overlapping binding sites, one at the entrance of the gorge, the peripheral anionic site (PAS), and one at the bottom of the gorge, the catalytic site (CAS) [15]. These two sites in AChE possess similar binding properties with a preference to bind cationic ligands. The natural substrate of AChE, the neurotransmitter acetylcholine, is a permanent cation and able to slide from its initial binding at the PAS to the bottom of the gorge where it is rapidly hydrolyzed by the catalytic triad (Ser203, His447 and Glu334, *h*AChE numbering is used throughout the text). Mosquitoes have two genes encoding AChE enzymes: *ace-1* and *ace-2* [16], where the enzyme encoded by *ace-1* (AChE1) appear to have the central catalytic function [17]. The mosquito AChEs has been less studied than the vertebrate AChE, although it has been shown to hydrolyze the same substrates with similar kinetic properties [18-21].

Two of the four chemical classes of insecticides recommended for IRS, namely organophosphates and carbamates, covalently inhibit the activity of AChE1. These insecticides are efficient at controlling disease-transmitting mosquitoes, however, their ability to inhibit AChE enzymes from many different species, including humans, give rise to safety concerns [22, 23]. In addition, some mosquito species have evolved a variant of the AChE1 enzyme featuring a point mutation in the active site (G122S in human AChE (*h*AChE), corresponding to G119S in *Torpedo californica* numbering) that confers resistance to existing organophosphate- and carbamate-based insecticides [24]. Consequently, new insecticides targeting AChE need to address both selectivity and target-site resistance. Encouraging results have been reported in the literature, including a number of covalent inhibitors of AChE1 displaying either selectivity for the mosquito enzyme over *h*AChE or retained activity on the resistant G122S mutant [20, 25-30]. To date, two different design strategies of selective covalent AChE1-inhibitors have been presented. In the first approach, a cysteine present near the active site gorge of AChE1, but not present in vertebrate AChE, has been targeted with covalent inhibitors [25, 28, 31, 32]. The second design strategy targets the Ser203 residue of the catalytic triad with covalent inhibitors, in a similar fashion as the currently approved and used insecticides. A variety of new potential inhibitors has been used to probe selectivity and potency profiles [20, 22, 26, 27, 29, 30, 33, 34].

Instead of aiming for new inhibitors that react covalently with AChE, our focus is on non-covalent inhibitors with the possibility to explore parts of the active site gorge located distant from the catalytic triad. In a recent differential high-throughput screen (HTS) against the AChE1 enzymes from *Anopheles gambiae* (*An. gambiae*; vector of the malaria parasite; *Ag*AChE1) and *Aedes aegypti* (*Ae. aegypti*; vector of the dengue, yellow fever, chikungunya, and Zika virus infection; *Aa*AChE1), we identified a number of non-covalent hits showing potential selectivity for the mosquito enzymes over the human enzyme [35]. Herein, one of the compound classes of AChE1 inhibitors discovered in the screen, *viz. N*-aryl-*N'*-ethyleneaminothioureas, has been explored by design, synthesis, and biochemical evaluation for inhibition of recombinant *Ag*AChE1, *Aa*AChE1, G122S-*Ag*AChE1 and *h*AChE. Furthermore, a selection of thioureas was investigated for their inhibitory effect on mosquito extract *in vitro* as well as their insecticidal effect on adult and larvae mosquitoes *in vivo*.

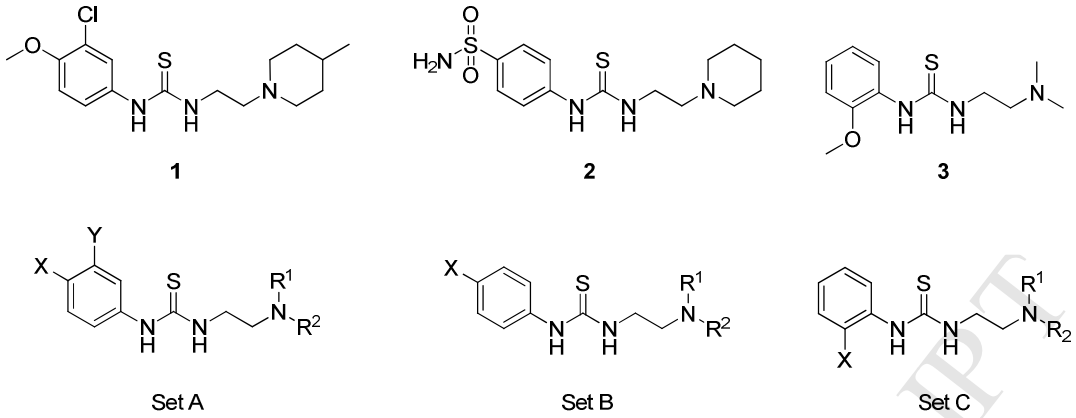
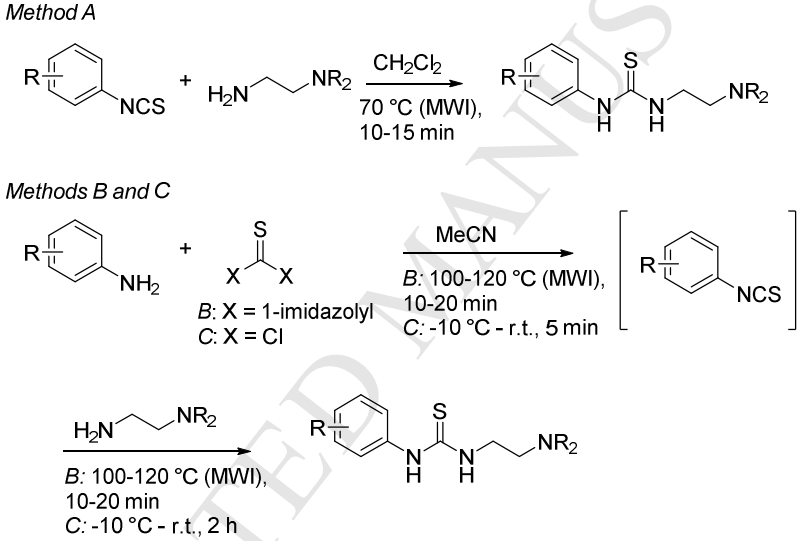


Figure 1. The chemical structures of the three hits (**1**-**3**) that formed the basis for the design of compounds in three sets of molecules (A-C). X and Y show variation sites of compounds in each of the three sets. The tertiary amine (NR^1R^2) in the hits was varied cross the three sets.



Scheme 1. Synthesis of *N*-aryl-*N*'-ethyleneamino substituted thioureas. MWI: microwave irradiation.

2. Results

2.1. Design and synthesis of thioureas

In a previously reported HTS against AChE1, we identified thiourea-based compounds that displayed potential selectivity towards inhibition of *Ag*/*Aa*AChE1 over inhibition of *h*AChE [35] (Figure 1). The compounds constituted a group of asymmetric thioureas, all carrying both an aryl substituent as well as one tertiary amine connected via a carbon chain linker. Three of these hits (**1**-**3**) constituted the basis for the design, resulting in three sets of molecules A-C (Figure 1). Set A consists of twelve molecules, and set B and C consist of five and four molecules, respectively (Tables 1-3).

Re-synthesis of the HTS hits **1**-**3** and synthesis of the designed *N*-aryl-*N'*-ethyleneamino substituted thioureas **4-21** were accomplished by one of three methods (A-C, Scheme 1) depending on commercial availability of the starting materials, ease of purification, and safety. Method A (Scheme 1) proved to be the most straightforward approach in case of commercially available phenylisothiocyanate, for which heating together with a primary amine for 10 or 15 minutes yielded the desired thioureas in good yields. The other

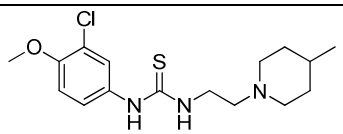
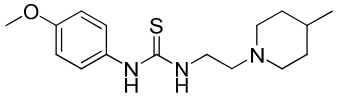
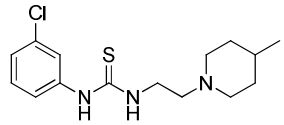
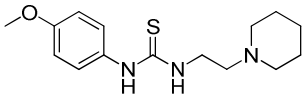
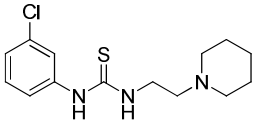
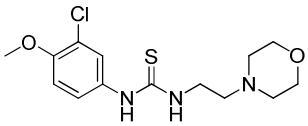
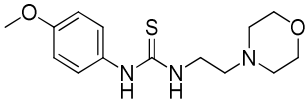
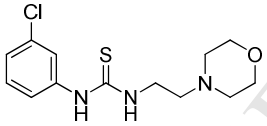
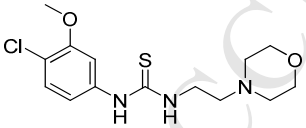
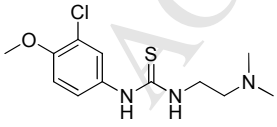
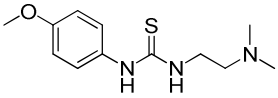
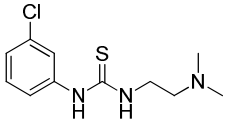
synthetic routes involved *in situ* formation of the isothiocyanate from the corresponding aniline, achieved by using thiocarbonyldiimidazole (TCDI, method B) or thiophosgene (method C) as the thiocarbonyl source. In a one-pot two-step reaction, the substituted aniline was first allowed to react with the thiocarbonyl source, after which a primary amine was added to give the thiourea. TCDI, a solid, is regarded as a safer and easier to handle alternative to thiophosgene. However, TCDI also has lower reactivity than thiophosgene and required heating to promote the reaction (Method B, Scheme 1), leading in some cases to the formation of undesired byproducts. Therefore, if TCDI gave poor results, thiophosgene was used instead (Method C, Scheme 1). Thiophosgene is a toxic, volatile liquid, but reacted quickly at room temperature, and in our hands produced less byproducts than TCDI, which facilitated the isolation and purification of the resulting thioureas. In case a synthesized thiourea proved difficult to isolate by crystallization or chromatography, salt formation was used as a means of purification (compounds **19** and **20**). The 21 synthesized thioureas were subjected to biochemical evaluations where the half-maximal inhibitory concentrations (IC_{50}) for *Ag*AChE1, *Aa*AChE1, *h*AChE and G122S-*Ag*AChE1 were determined.

2.2. Structure-activity relationships of AChE1 inhibition

The potencies of the compounds were assessed by determining their IC_{50} values using the colorimetric Ellman assay and four different AChEs (*i.e Ag*AChE1, *Aa*AChE1, *h*AChE and G122S-*Ag*AChE1). The compounds potencies varied several orders of magnitude (Tables 1-3), with IC_{50} values ranging from 90 nM to inactive (IC_{50}>1 mM) for *Ag*AChE1 and/or *Aa*AChE1. Seven compounds displayed promising AChE1 potencies with IC_{50} values below 20 µM, five had IC_{50} values of 20-100 µM, while nine compounds were weak inhibitors (IC_{50} values > 100 µM) or inactive. The inhibition profiles based on the IC_{50} values for *Ag*AChE1 and *Aa*AChE1 showed that the two mosquito enzymes behaved in a very similar manner when being probed with this class of compounds. The three HTS hits **1**-**3** all inhibited *Ag*AChE1 and *Aa*AChE1 in a dose-dependent manner (Figure 2a-c, Tables 1-3). Hit **1,** which displayed 90% inhibition of AChE1 in the HTS, proved to be the most potent inhibitor of the three with IC_{50} values of 0.93 and 0.63 µM for *Ag*AChE1 and *Aa*AChE1, respectively (Figure 2a). Compounds **2** and **3**, which in the HTS showed 70% and 50% inhibition of AChE1, respectively, were also fairly potent inhibitors with a factor ten higher IC_{50} values compared to **1** (Figure 2b-c, Table 2 and 3).

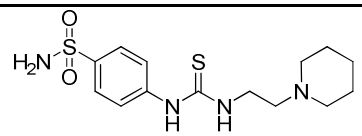
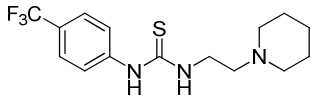
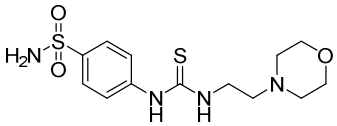
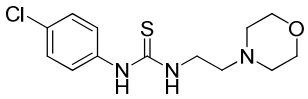
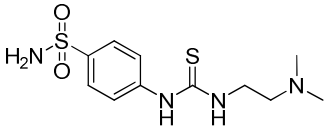
The inhibitory effect of **1** in set A was very sensitive to changes in the 3-chloro-4-methoxy substitution pattern on the phenyl ring of **1**; a large drop in potency was seen when exchanging any of the substituents for hydrogens, independent of the tertiary amines (Table 1). Also, switching the position of the 3-chloro-4-methoxy substituents on the phenyl ring to 4-chloro-3-methoxy resulted in a substantial reduction in inhibitory effect (**11**). Variations of the tertiary amines of the analogues in set A were more tolerated; replacing the 4-methylpiperidine moiety for a the more polar morpholine resulted in the most potent inhibitor (**8**) with IC_{50} values of 120 nM and 90 nM for *Ag*AChE1 and *Aa*AChE1, respectively, while the dimethyl analogue **12** led to decreased IC_{50} values. Interestingly, the SAR of **2** in set B suggests a different AChE1 binding mode of the 4-sulfamoyl phenyl thioureas compared to the analogues of **1** (Table 2), as compound **16** with the morpholine moiety shows very low inhibitory effects of AChE1. It also seems, within this limited set of substituents on the phenyl ring, that inhibitory effect of the 4-sulfamoyl substituent could be due to hydrogen bond donor and acceptor capabilities, and not electronic effects of the aromatic ring, since both electron donating (methoxy) and electron withdrawing (trifluoromethyl) substituents resulted in reduced inhibition. The SAR of the ortho substituted phenyls in the limited set C, suggests that small amines are preferred for potent inhibitors, in contrast to the analogs in sets A-B (Table 3), as the dimethyl amine moiety was more active than the piperidine- and morpholine moieties. These results suggests a third binding mode of the thiourea inhibitors.

Table 1. Chemical structures and IC_{50} values for compounds in set A.

No	Structure	IC_{50}[a] (μM)			S.R.[b]	IC_{50}[a] (μM)
		*Ag*AChE1	*Aa*AChE1	*h*AChE	*h*AChE/ AChE1	G122S-*Ag*AChE1
1		0.96 (0.67-1.4)	0.63 (0.27-1.5)	12 (8.5-17)	13	24 (9.6-58)
4		>200	>200	>200	na[d]	>200
5		>200	>200	>200	na[d]	>200
6		29 (19-45)	28 (18-44)	120 (82-160)	4	94 (58-150)
7		140 (110-180)	120 (86-170)	190 (121-302)	1	22 (16-30)
8		0.12 (0.11-0.14)	0.090 (0.083-0.098)	14 (13-15)	117	84 (33-212)
9		11 (8.7-13)	11 (9.2-14)	>200	>18	>200
10		68 (41-110)	53 (45-62)	>200	>3	>200
11		>200	>200	>200	na[d]	>200
12		18 (17-20)	16 (14-19)	46 (39-53)	3	17 (6.0-49)
13		>200	>200	>200	na[d]	>200
14		96 (85-110)	83 (70-99)	140 (120-170)	1	>200

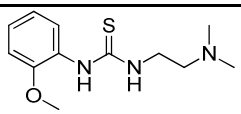
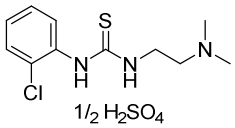
[a] IC_{50} values were determined using 2-3 replicates, the 95% confidence interval is given in parentheses. [b] S.R. = selectivity ratio; were computed by taking the compound's *h*AChE IC_{50} value divided by the higher IC_{50} value for *Ag* or *Aa.* [c] inactive up to 1 mM. [d] nd = not determined. [e] na = not applicable.

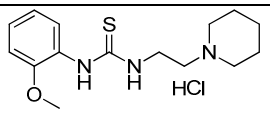
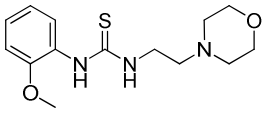
Table 2. Chemical structures and IC_{50} values for compounds in set B.

No	Structure	IC_{50}[a] (μM)			S.R.[b]	IC_{50}[a] (μM)
		*Ag*AChE1	*Aa*AChE1	*h*AChE	*h*AChE/ AChE1	G122S-*Ag*AChE1
2		7.8 (6.9-8.7)	5.5 (4.8-6.2)	90 (72-110)	12	33 (17-65)
15		>200	>200	>200	na[d]	>200
16		>200	>200	>200	na[d]	Inactive[c]
17		Inactive[c]	Inactive[c]	Inactive[c]	na[d]	Inactive[c]
18		74 (66-84)	56 (43-73)	>200	>2.7	Inactive[c]

[a] IC_{50} values were determined using 2-3 replicates, the 95% confidence interval is given in parentheses. [b] S.R. = selectivity ratio; were computed by taking the compound's *h*AChE IC_{50} value divided by the higher IC_{50} value for *Ag* or *Aa.* [c] inactive up to 1 mM. [d] nd = not determined. [e] na = not applicable.

Table 3. Chemical structures and IC_{50} values for compounds in set C.

No	Structure	IC_{50}[a] (μM)			S.R.[b]	IC_{50}[a] (μM)
		*Ag*AChE1	*Aa*AChE1	*h*AChE	*h*AChE/ AChE1	G122S-*Ag*AChE1
3		7.1 (6.5-7.6)	6.0 (5.6-6.5)	120 (100-130)	17	50 (29-86)
19	 $1/_2\ H_2SO_4$	17 (14-22)	14 (13-16)	>200	>12	>200

20		29 (26-33)	22 (19-24)	32 (29-35)	1	66 (20-220)
21		>200	>200	>200	na[d]	Inactive[c]

[a] IC_{50} values were determined using 2-3 replicates, the 95% confidence interval is given in parentheses. [b] S.R. = selectivity ratio; were computed by taking the compound's *h*AChE IC_{50} value divided by the higher IC_{50} value for *Ag* or *Aa*. [c] inactive up to 1 mM. [d] nd = not determined. [e] na = not applicable.

2.3. Structure-selectivity relationship for AChE1 versus *h*AChE

The biochemical evaluation of the compounds' potency for *Ag*AChE1, *Aa*AChE1 and *h*AChE showed potent AChE1 inhibitors that also displayed selectivity for AChE1 over *h*AChE (Table 1). For example, the most potent AChE1 inhibitor (**8**) had a reduced potency of a factor 100 for *h*AChE (Figure 2d). Overall, the compounds showed a much lower inhibitory effect on *h*AChE compared to the mosquito enzymes. Only two compounds had IC_{50} values below 20 µM for *h*AChE (compared to seven for AChE1) and the most potent *h*AChE inhibitor (**1**) had an IC_{50} of 12 µM. The selectivity ratios based on the IC_{50} values (S.R.; *h*AChE/AChE1) ranged from 1 to over 100 (Tables 1-3).

In set A, the largest SSR effect was seen for the variations in the tertiary amine moiety. Three inhibitors all having the same 3-chloro-4-methoxy phenyl ring but with different tertiary amines (**1**, **8** and **12**) differed significantly in their S.R. values; the 4-methylpiperidine moiety (**1**) resulted in S.R. of 13, while the morpholine (**8**) and the dimethylamino (**12**) moieties gave S.R. values of 117 and 3, respectively. The increased S.R. for the morpholine analogue **8** compared to **1** was due to the increased potency of **8** for AChE1, which was not seen for *h*AChE. The same trend was observed for all morpholine analogues (**8**, **9** and **10**). The introduction of the small dimethylamine moiety (**12**) instead of 4-methylpiperidine (**1**) led to a smaller loss of potency for the human form compared to the mosquito form (1/4 vs. 1/20), resulting in decreased selectivity. In addition, the substituents on the phenyl ring were of importance for the selectivity as seen in sets B and C. The 4-sulfamoyl substituent in **2** showed a higher selectivity toward AChE1 over *h*AChE (S.R. = 12) than its corresponding methoxy analogue (**6**; S.R. = 4). In set C, the ortho-methoxy phenyl thiourea with dimethylamine as tertiary amine had a S.R. of 17, while the dimethylamine analogues of set A showed low or no selectivity towards AChE1 (**12**, **13**, **14**). It was also clear that replacing the dimethylamine moiety for a piperidine lead to loss of selectivity (*cf.* **3** vs. **20**). These results clearly demonstrate that it is possible to design and synthesize selective non-covalent AChE1 inhibitors.

2.4. Structure-activity and structure-selectivity relationships of the G122S-*Ag*AChE1 mutant

Our data clearly show that the compounds in sets A-C had a reduced potency of G122S-*Ag*AChE1 compared to wild type; only five compounds had IC_{50} values equal or below 50 µM (**1**, **8**, **12**, **2** and **3**), compared to seven compounds displaying IC_{50} values below 20 µM on wild type *Ag*AChE1. The overall G122S-*Ag*AChE1 inhibition profiles of the compounds were more similar to the effect on *h*AChE than to *Ag/Aa*AChE1. For example, the morpholine analogues in set A did not lead to an increase in potency compared to the methylpiperidine analogues (*cf*. **1** vs. **8**). Nevertheless, three of the compounds (**12**, **2** and **3**) showed moderate selectivity towards G122S-*Ag*AChE1 compared to *h*AChE (2-3 times difference). Both **12** and **3** have the smaller dimethylamine moiety, which seems to result in stronger inhibition effect on G122S-*Ag*AChE1 compared to *h*AChE. Also, the 4-sulfamoyl substituent of **2** shows promising selectivity towards G122S-*Ag*AChE1 over *h*AChE. Interestingly, one compound (**7**) displayed stronger potency on the G122S mutant than on either of the wild type AChE1 tested or the *h*AChE.

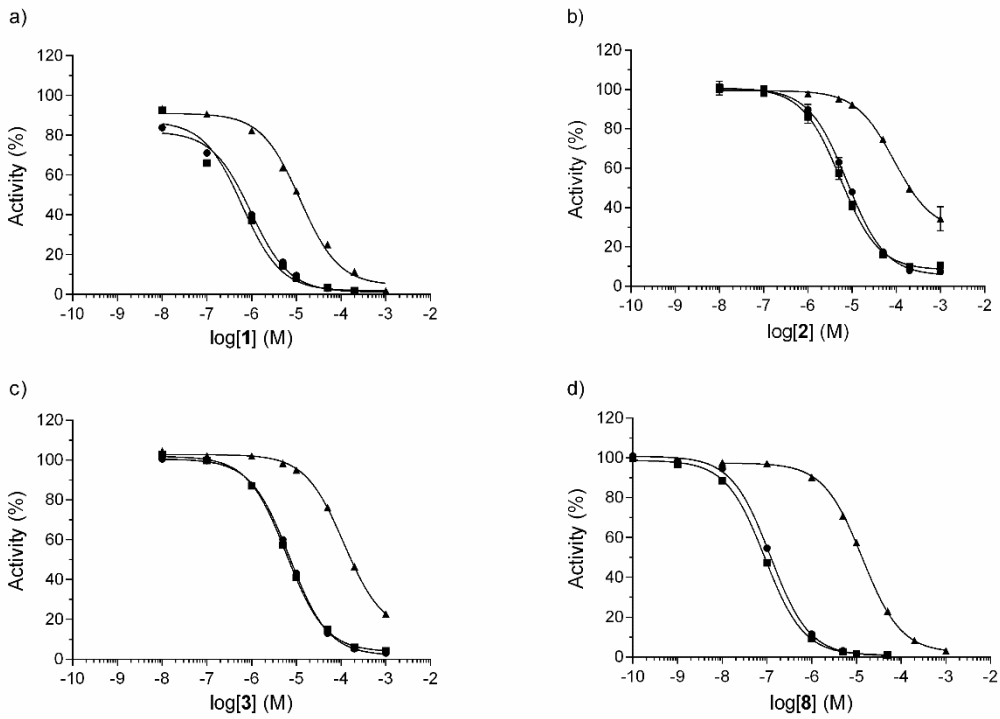


Figure 2. Dose-response curves showing the relative inhibition of recombinant *Ag*AChE1 (dots), *Aa*AChE1 (squares) and *h*AChE (triangles) at different concentrations of compounds **1**-**3** (a-c) and **8** (d), where the highest compound concentration used was 1 mM. IC_{50} values for inhibition of *Ag*AChE1, *Aa*AChE1 and *h*AChE are presented in Tables 1-3.

2.5. Inhibitory effect of thioureas on mosquito extract

The inhibitory effect of three compounds (**8**, **3** and **13**) with different *in vitro* IC_{50} values for recombinant *Aa*AChE1 was subsequently tested *ex vivo* on pooled extracts from 30 adult female *Ae. aegypti* mosquitoes (Figure 3). Homogenized mosquito extracts from either the head or the rest of the body were used as the enzyme source. The compounds' inhibitory effect observed in these mosquito extract experiments corresponded well with the IC_{50} values determined on recombinant expressed *Aa*AChE1. The IC_{50} value of **8**, being the most potent and selective compound in all sets, was determined to 0.19 (0.12-0.29) µM and 0.15 (0.076-0.30) µM for head and body extract respectively (*cf.* IC_{50} value of 0.090 (0.083-0.098) µM for recombinant *Aa*AChE1). Compound **3** displayed IC_{50} values of 7.1 (4.1-12) µM and 7.6 (5.0-11) µM for head and body extract respectively, in comparison to 6.0 (5.6-6.5) µM for recombinant *Aa*AChE1. The inactive compound **13** was shown to be inactive up to 1 mM also on mosquito extracts. Our results show no significant difference between enzyme extracted from the mosquito head or from the body.

The good agreement of the IC_{50} values for recombinant AChE1 and for mosquito extract shows that the Ellman assay using recombinant AChE is a valid model for acetylcholine hydrolysis in *Ae. aegypti*. The results also suggest that other enzymes, on the timescale of the experiment, do not degrade either acetylthiocholine or the tested compound.

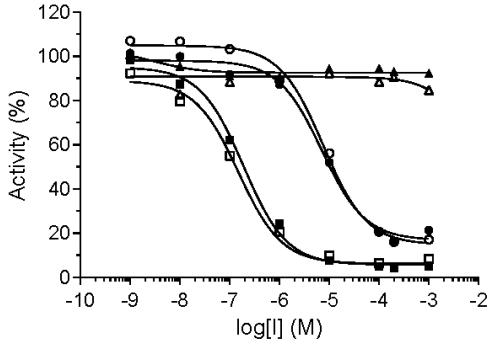


Figure 3. Dose-response curves showing the inhibition of esterase activity obtained from homogenized adult mosquitoes of the head (filled symbols) and body (empty symbols) for compound **8** (squares), compound **3** (circles) and compound **13** (triangles).

2.6. Insecticidal effect of thioureas exposed to mosquitoes

The three compounds assayed in the mosquito extract experiment (**8**, **3** and **13**) were also tested for their insecticidal effect on living mosquitoes. *In vivo* experiments were performed on five days old adults and 3rd instar larvae of *An. gambiae* and *Ae. aegypti*. The *in vivo* potency of the compounds was investigated through topical application on adults and treated water for compound exposure to larvae. All three thioureas killed adult *An. gambiae*, although very high concentrations of the compounds were needed (Tables 4 and 5). The most efficient compound, **8**, showed 70% mortality of *An. gambiae* at a 1 nmol dose, which can be compared to the commercial insecticide propoxur with a topical LD_{50} of 0.015 nmol/mosquito [26]. The thioureas **3** and **13** had a less potent effect on *An. gambiae* with approximately 55% mortality at a 10 nmol dose. The potency of these inhibitors on *Ag*AChE1 differs; compound **3** has an IC_{50} value of 6 µM, while **13** showed only 30% inhibition at 1 mM. No effect was seen on adult *Ae. aegypti* for any of the tested compounds at the tested concentrations. A more prominent effect was seen on *Ae. aegypti* larvae where exposure of compound **8** at 500 µM gave 97% mortality. The larvicidal effect was even stronger on *An. gambiae* larvae, where exposure of compound **8** at 300 µM gave 100% mortality. The other tested compounds did not have any effect on the larvae.

Table 4. Mortality of adult mosquitoes 24 hours after topical applications of thioureas[a]

Compound	*An. gambiae* (% mortality)				*Ae. aegypti* (% mortality)			
	0.01 nmol	0.2 nmol	1 nmol	10 nmol	0.01 nmol	0.2 nmol	1 nmol	10 nmol
8	0	22	70	*n.d.*[b]	-	5	0	*n.d.*[b]
3	-	16	0	57	-	8	10	10
13	-	0	32	55	-	1	0	12

[a] 68 and 40 mosquitoes on average for each treated and control experiment, respectively. The mortality was adjusted based on acetone controls using Abbott's formula[36]. Compounds were administered in an acetone solution (0.1 µl/mosquito). [b] *n.d.* = not determined due to solubility issues.

Table 5. Mortality of larvae mosquitoes 24 hours after treatments of thioureas

Compound	*An. gambiae* (% mortality)[a, b]			*Ae. aegypti* (% mortality)[a, b]		
	50 µM	300 µM	500 µM	50 µM	300 µM	500 µM
8	1	100	100	8	64	97
3	5	-	3	7	-	5
13	4	-	3	4	-	1

[a]75 and 50 larvae for each treated and control experiment, respectively, < 5 % mortality in controls; [b] Compounds dissolved in de-chlorinated tap water to the given concentration in 100 mL.

3. Discussion

Analogues were made of the hits **1**-**3** to give sets A-C, with local variations around the aromatic moieties, and a selection of amines included in all three sets. It was clear from the SAR analysis that the AChE1 inhibitory effect of thioureas was dependent on specific combinations of the aromatic moiety and the amine. For instance, the morpholine was preferred in combination with the 3-chloro-4-methoxy phenyl but not with the ortho-methoxy phenyl (*cf.* **8** and **3**). The best inhibition effect for the ortho-methoxy phenyl was instead seen in combination with dimethyl amine (*cf.* **3** and **12**). The lack of common SAR suggests that the three most potent inhibitors, one from each set of compounds, have three different binding modes in the active site gorge of AChE1. Furthermore, based on the SAR, the aromatic moiety appears to dictate the binding mode of the molecule, and thus the positioning of the amines is dependent on that binding mode. Comparisons of the thioureas with other known AChE inhibitors [35, 37, 38], suggests that the aromatic moiety bind in PAS and the amine moiety is directed towards CAS. One hypothesis would then be that it is aromatic interactions and hydrogen bonding between the aromatic moieties of **8**, **2** and **3** and residues in PAS that position the inhibitors **8**, **2** and **3** differently in the active site gorge.

The thioureas had generally a much lower potency on the human isoform, which opens up for selective inhibitors towards the mosquito enzyme. Unfortunately, the inhibitors were also less effective on the resistance-conferring mutant, an important aspect to consider in potentially further chemical optimizations into insecticides. Still, the SAR and SSR analysis (*c.f.* **2** and **3**) reveal potential to develop divergent SARs for human AChE and the G122S-*Ag*AChE1 mutant while maintaining efficient inhibition of the wild type, an acknowledged challenge [29, 30]. We have previously published a potent AChE1 inhibitor from a different compound class that also efficiently inhibit the resistance-conferring mutant *and* is selective over the human AChE [35].

The inhibition effects of the thioureas based on IC_{50} values observed for recombinant AChE1 were confirmed *ex vivo* using mosquito extract. Although few compounds were tested, the *ex vivo* experiments showed that the thioureas also efficiently inhibited AChE1 from mosquitoes, and thus indicated their potential as insecticides. Indeed, the most potent thiourea, **8**, killed both adults and larvae of the *An. gambiae* species at high doses. Similarly, compounds **3** and **13** also had mortal effects on adult *An. gambiae* mosquitoes, although at even higher doses. AChE1 enzymes from *An. gambiae* and *Ae. aegypti* appear to have very similar functionally and inhibitory properties *in vitro* [21, 35]. Nevertheless, in our *in vivo* studies, we observed differences between the inhibitors' effect on the two mosquito species, as none of the thiourea inhibitors tested on *Ae. aegypti* showed adult mortality, and only the most potent compound **8** had an insecticidal effect on *Ae. aegypti* larvae. The difference in inhibitor mortality of *An. gambiae* and *Ae. aegypti* is most likely due to differences in the pharmacokinetic properties of the tested compounds in the two mosquito species.

The thiourea-based inhibitors are among the first non-covalent selective inhibitors of AChE1 shown to kill mosquitoes. The discrepancy seen between the *in vitro* potency and *in vivo* efficacy may be due to issues

related to penetration through the mosquitoes´ exoskeleton or to metabolism, an issue suggested also for covalent inhibitors of AChE1 tested on mosquitoes [20, 26, 27, 29]. Thus, future development should pay attention not only to *in vitro* and *in vivo* potency, but also include pharmacokinetic properties, such as penetration and metabolism.

4. Conclusions

New tools for vector control of disease-transmitting mosquitoes are needed to successfully combat diseases like malaria and dengue. The results presented here are encouraging for further development of non-covalent inhibitors of AChE1 towards the discovery of new insecticides. Highly potent and selective AChE1 inhibitors were designed that displayed *in vivo* insecticidal activity in mosquitoes of the *An. gambiae* and *Ae. aegypti* species. The SAR for inhibition of AChE1 was complex and could be explained by multiple binding poses and/or multiple key interactions formed by the analogues. The complex molecular recognition of AChEs was also evident by the single point mutation G122S of *Ag*AChE1 that not only led to loss in potency of the thioureas, but also led to a different SAR. The present work highlight some of the challenges associated with the discovery and development of insecticide-candidates of AChE1 inhibitors; potency to wild type and mutant enzyme, selectivity over higher species as well as optimization of the pharmacokinetic properties. In this multi-objective task, we believe that careful designs of sets of compounds, guided by SARs and SSRs of AChE1 and related targets, have an important role to play.

5. Experimental section

5.1. General aspects of the synthesis of thioureas

All reactions were carried out under inert atmosphere unless otherwise stated. Dry CH_2Cl_2 was obtained by passing through neutral alumina using a solvent drying system, and was freshly collected prior to the reaction, and MeCN was dried over 4Å molecular sieves for >48 h. All microwave reactions were carried out in a monomode reactor using Smith process vials sealed with a Teflon septa and an aluminum crimp top. The temperature was measured with an IR sensor and reaction times refer to the irradiation time at the target temperature. Reactions were monitored using TLC (silica gel matrix, layer thickness 200 µm, particle size 25 µm) with UV-detection (254 nm) or developed using $KMnO_4$ solution, or using LC/MS. Flash column chromatography (eluents given in brackets) was performed on normal phase silica gel (Merck, 60 Å, 40-63 µm). High resolution mass spectrometry (HRMS) data was obtained on Agilent Technologies 6230 TOF LC/MS in ESI mode. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 or DRX-600 instrument at 298 K in $CDCl_3$ using residual $CHCl_3$ (δ_H = 7.26 ppm) or $CDCl_3$ (δ_C = 77.16 ppm) as an internal standard, $(CD_3)_2SO$ using residual $(CD_3)(CD_2H)SO$ (δ_H = 2.50 ppm) or $(CD_3)_2SO$ (δ_C = 39.52 ppm) as an internal standard, or CD_3OD using residual CD_2HOD (δ_H = 3.31 ppm) or CD_3OD (δ_C = 49.0 ppm) as an internal standard. ^{19}F NMR spectra were calibrated against an external standard of CF_3COOH in DMSO-*d6* (δ_F = −76.55 ppm).

5.2. Synthesis of building blocks

tert-Butyl (2-(4-methylpiperidin-1-yl)ethyl)carbamate. 2-Bromoethylamine hydrobromide (1.11 g, 5.43 mmol, 1.1 eq.) was added in one portion to di-tert-butyl dicarbonate (1.07 g, 4.88 mmol, 1.0 eq.) in 26 ml of dried CH_2Cl_2 at 0 ^{o}C while stirring. Triethylamine (1.02 ml, 7.29 mmol, 1.5 eq.) was added dropwise to the mixture, and upon complete addition the reaction mixture was stirred at rt for 24 h. The mixture was diluted with CH_2Cl_2, washed twice with NH_4Cl (aq., sat.), $NaHCO_3$ (aq., sat.) and brine, and the aqueous layers were extracted once with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced

pressure. The resulting crude product showed remaining di-tert-butyl dicarbonate and was dissolved in CH_2Cl_2, washed with water, dried over Na_2SO_4, filtered and concentrated. The resulting clear oil was dissolved in 10 ml CH_2Cl_2 and 4-methylpiperidine (1.12 ml, 9.76 mmol, 2.0 eq.) was added dropwise at rt while stirring. The reaction mixture was heated at reflux for 20 h. After being allowed to attain rt, the reaction mixture was diluted with CH_2Cl_2, washed once with water followed by brine, and the aqueous layers were extracted once with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. Purification by column chromatography (CH_2Cl_2:MeOH 90:10) gave the title compound (801 mg, 68% yield over two steps) as a clear oil, which solidified upon cooling. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.06 (br s, 1H), 3.22-3.18 (m, 2H), 2.84-2.91 (m, 2H), 2.42-2.39 (m, 2H), 1.93 (dt, J_1 = 11.9 Hz, J_2 = 1.9 Hz, 2H), 1.60-1.57 (m, 2H), 1.42 (s, 9H), 1.37-1.28 (m, 1H), 1.27-1.15 (m, 2H), 0.89 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.1, 79.1, 57.6, 53.9, 37.4, 34.3, 30.9, 28.6, 22.0.

2-(4-Methylpiperidin-1-yl)ethan-1-amine. *tert*-Butyl (2-(4-methylpiperidin-1-yl)ethyl)carbamate (759 mg, 3.13 mmol) was stirred in 3 ml of HCl (aq., 3 M) at rt for 20 h. The mixture was made basic by addition of NaOH (aq., 2 M) before being extracted three times with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give the title compound (306 mg, 69% yield) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.81-2.79 (m, 2H), 2.74 (t, J = 6.4 Hz, 2H), 2.33 (t, J = 6.4 Hz, 2H), 1.88 (t, J = 11.5 Hz, 2H), 1.58-1.54 (m, 2H), 1.50 (br s, 2H), 1.34-1.26 (m, 1H), 1.23-1.13 (m, 2H), 0.87 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 61.8, 54.3, 39.2, 34.5, 30.9, 22.0.

4-Chloro-3-methoxyaniline. Pd(C) (10%, 142 mg, 0.13 mmol, 0.1 eq. Pd) was added to a solution of 1-chloro-2-methoxy-4-nitrobenzene (250 mg, 1.33 mmol, 1.0 eq.) in 10 ml of EtOAc. The vessel was evacuated, backfilled with H_2, fitted with a H_2 balloon, and stirred for 16 h. The suspension was filtered through a pad of Celite and concentrated. NMR indicated 5-10% of the dehalogenated product; therefore, the crude product mixture was recrystallized from heptane with a minimal amount of CH_2Cl_2 to give the title compound as white crystals (111 mg, 0.70 mmol, 53% yield). The ^{1}H spectrum was in accord with literature data [39].

5.3. General procedure for method A for synthesis of thioureas

To the appropriate phenyl isothiocyanate (1 eq) in dry CH_2Cl_2 (0.1 mmol/ml) the corresponding amine (1-1.2 eq) was added dropwise at rt while stirring. The reaction mixture was heated at 70 °C for 15 minutes in a sealed vial purged with N_2 using microwave irradiation. After attaining rt, the reaction mixture was diluted with EtOAc and washed with sat $NaHCO_3$ (aq) followed by brine and the aqueous layers were extracted with EtOAc x3. The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The crude product was purified by recrystallization from heptane:CH_2Cl_2 (5:1) and the formed crystals were washed with a small amount of heptane.

5.4. General procedure for method B for synthesis of thioureas

To thiocarbonyldiimidazole (1 eq.) in dried MeCN (0.1-0.2 M), the appropriate aniline (1 eq.) was added. The reaction vial was purged with N_2, sealed, and stirred at 100 °C for 10 minutes in a microwave reactor. The appropriate alkylamine was added, and the reaction vessel was heated to 100 °C for 10 minutes. After attaining rt, the reaction mixture was diluted with EtOAc and washed three times with $NaHCO_3$ (aq., sat.), dried over Na_2SO_4, filtered and concentrated. Recrystallization from an appropriate solvent or solvent mixture gave the desired compound.

5.5. General procedure for method C for synthesis of thioureas

To thiophosgene (1 eq.) in $CHCl_3$ (0.5-1 M), the appropriate aniline (1 eq.) was added. After adding Et_3N (2 eq.), the appropriate alkylamine (1 eq.) was added. After stirring for 2 h, the reaction mixture was concentrated in vacuo, dissolved in EtOAc, washed three times with $NaHCO_3$ (aq., sat.), dried over Na_2SO_4, filtered and concentrated. Recrystallization from an appropriate solvent or solvent mixture gave the desired compound.

5.6. Synthesis of thioureas 1-21

1-(3-Chloro-4-methoxyphenyl)-3-(2-(4-methylpiperidin-1-yl)ethyl)thiourea (1). Method B. 3-Chloro-4-methoxyaniline (45 mg, 0.29 mmol, 1.0 eq.) was added in one portion to 1,1'-thiocarbonyldiimidazole (51 mg, 0.29 mmol, 1.0 eq.) in 1 ml of dried MeCN at rt while stirring. The reaction mixture was heated to 100 °C for 10 min using microwave irradiation. 2-(4-Methylpiperidin-1-yl)ethan-1-amine (*vide supra*, 41 mg, 0.29 mmol, 1.0 eq.) in 0.5 ml of dried MeCN was added dropwise and the reaction mixture was heated at 100 °C for an additional 10 min using microwave irradiation. The resulting brown solution was cooled to rt upon which a white precipitation formed. The precipitate was collected by filtration, and washed with MeCN and heptane to give thiourea **1** as off-white crystals (41 mg, 42% yield). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.56 (br s, 1H), 7.50 (s, 1H), 7.45 (br s, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 7.11 (d, *J* = 8.8 Hz, 1H), 3.83 (s, 3H), 3.54-3.48 (m, 2H), 2.83-2.77 (m, 2H), 2.44-2.42 (m, 2H), 1.91-1.88 (m, 2H), 1.55 (d, *J* = 12.1 Hz, 2H) 1.33-1.29 (m, 1H), 1.10-1.04 (m, 2H), 0.87 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (150 MHz, DMSO-*d6*) δ 180.2, 151.7, 132.3, 125.5, 124.9, 120.5, 112.7, 56.2, 56.1 (br), 53.2, 41.2, 40.0, 33.9, 30.3, 21.8. HRMS calcd for $[C_{16}H_{24}ClN_3OS+H]^+$ 342.1402, found 342.1402.

1-(2-(Piperidin-1-yl)ethyl)-3-(4-sulfamoylphenyl)thiourea (2). Modified method A. 1-(2-Aminoethyl)piperidine (105 mg, 0.49 mmol, 1.0 eq.) was added to 4-isothiocyanatobenzenesulfonamide[40] (0.07 ml, 0.49 mmol, 1.0 eq.) in 3 ml of CH_2Cl_2 in a thick-walled vial. The vial was sealed and heated to 70 °C for 10 min in a microwave reactor. The white suspension was concentrated in vacuo, and carefully recrystallized from EtOH/water (6:1.5 ml) to give thiourea **2** as white crystals (104 mg, 0.304 mmol, 62% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 10.00 (br s, 1H), 7.91 (br s, 1H), 7.73 (d, *J* = 8.6 Hz, 2H), 7.68 (d, *J* = 8.6 Hz, 2H), 7.27 (s, 2H), 3.69-3.44 (m, 2H), 2.48-2.43 (m, 2H), 2.43-2.26 (m, 4H), 1.61-1.45 (m, 4H), 1.45-1.31 (m, 2H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 179.9, 142.7, 138.4, 126.3, 121.2, 56.4, 53.9, 41.2, 25.5, 24.0. HRMS calcd for $[C_{14}H_{22}N_4O_2S_2+H]^+$ 343.1257, found 343.1254.

1-(2-(Dimethylamino)ethyl)-3-(2-methoxyphenyl)thiourea (3). Method C. 2-Methoxyaniline (1.13 ml, 10.0 mmol, 1.0 eq.) was added to thiophosgene (0.77 ml, 10.0 mmol, 1.0 eq.) in 20 ml of $CHCl_3$. The formed thick suspension was stirred for 5 minutes, after which *N*,*N*-dimethylethylenediamine (1.09 ml, 10.0 mmol, 1.0 eq.) was added over 5 minutes. Et_3N (2.8 ml, 20 mmol, 2.0 eq.) was added, and the suspension cleared. The reaction was stirred at r.t. for 2 h, and was then concentrated in vacuo. The crude reaction mixture was partitioned between EtOAc and $NaHCO_3$ (aq., sat.), the phases were separated, and the organic phase was washed twice with NaHCO3 (aq., sat.). The organic phase was dried over Na_2SO_4, filtered and concentrated to give a brown oil. The oil was dissolved in 6 ml of toluene/heptane 1:1, and upon slow cooling and swirling, thiourea **3** precipitated as an off-white powder (1.430 g, 5.64 mmol, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 323 K) δ 7.88 (br s, 1H), 7.37 (d, *J* = 7.2 Hz, 1H), 7.19-7.13 (m, 1H), 6.95 (br s, 1H), 6.97-6.89 (m, 2H), 3.83 (s, 3H), 3.69-3.60 (m, 2H), 2.46 (t, *J* = 5.8 Hz, 2H), 2.8 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$, 323 K) δ 180.7, 152.1, 126.8, 126.5, 124.2, 120.9, 111.9, 57.5, 55.8, 45.0, 43.1. HRMS calcd for $[C_{12}H_{19}N_3OS+H]^+$ 254.1322, found 254.1323.

1-(4-Methoxyphenyl)-3-(2-(4-methylpiperidin-1-yl)ethyl)thiourea (4). Following the general method A and starting from 4-metoxyphenyl isothiocyanate (48 µl, 0.35 mmol) and 2-(4-methyl-1-piperidinyl)ethanamine (50 mg, 0.35 mmol), thiourea **4** was obtained as an off-white solid (86 mg, 80% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.47 (br s, 1H), 7.27 (br s, 1H), 7.22 (d, *J* = 8.9 Hz, 2H), 6.90 (d, *J* = 8.9 Hz, 2H), 3.74 (s, 3H), 3.52-3.45 (m, 2H), 2.77 (d, *J* = 9.8 Hz, 2H), 2.41 (t, *J* = 6.2 Hz, 2H), 1.89 (t, *J* = 11.2 Hz, 2H), 1.54 (d, *J* = 12.5, 2 H), 1.34-1.23 (m, 1H),

1.01 (dq, J_1 = 3.7 Hz, J_2 = 12.5 Hz, 2H), 0.86 (d, J = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 180.2, 156.7, 131.4, 126.0, 114.1, 55.9, 55.3, 53.1, 41.2, 34.0, 30.3, 21.9. HRMS calcd for $[C_{16}H_{25}N_3OS+H]^+$ 308.1791, found 308.1782.

1-(3-Chlorophenyl)-3-(2-(4-methylpiperidin-1-yl)ethyl)thiourea (5). Following the general method A and starting from 3-chlorophenyl isothiocyanate (46 µl, 0.35 mmol) and 2-(4-methyl-1-piperidinyl)ethanamine (50 mg, 0.35 mmol), thiourea **5** was obtained as an off-white solid (81 mg, 74% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.82 (br s, 1H), 7.74 (br s, 1H), 7.70 (s, 1H), 7.33-7.32 (m, 2H), 7.15-7.13 (m, 2H), 3.55 (m, 2H), 2.84 (d, J = 9.6 Hz, 2H), 2.46 (t, J = 6.2 Hz, 2H), 1.91 (t, J = 10.9 Hz, 2H), 1.58 (d, J = 11.3, 2 H), 1.37-1.27 (m, 1H), 1.17-1.09 (m, 2H), 0.88 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 179.8, 140.0, 132.8, 130.3, 123.5, 121.8, 120.8, 56.1, 53.3, 41.3, 33.9, 30.3, 22.9. HRMS calcd for $[C_{15}H_{22}ClN_3S+H]^+$ 312.1296, found 312.1288.

1-(4-Methoxyphenyl)-3-(2-(piperidin-1-yl)ethyl)thiourea (6). Following the general method A and starting from 4-metoxyphenyl isothiocyanate (69 µl, 0.5 mmol) and 1-(2-aminoethyl)piperidine (71 µl, 0.5 mmol), thiourea **6** was obtained as a light brown solid (103 mg, 70% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.46 (br s, 1H), 7.29 (br s, 1H), 7.22 (d, J = 8.9 Hz, 2H), 6.90 (d, J = 8.9 Hz, 2H), 3.73 (s, 3H), 3.55-3.45 (m, 2H), 2.40 (t, J = 6.2 Hz, 2H), 2.36-2.29 (m, 4H), 1.47-1.42 (m, 4H), 1.38-1.34 (m, 2H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 180.3, 156.6, 131.5, 125.8, 114.1, 56.5, 55.3, 53.8, 41.2, 25.6, 24.0. HRMS calcd for $[C_{15}H_{23}N_3OS+H]^+$ 294.1635, found 294.1628.

1-(3-Chlorophenyl)-3-(2-(piperidin-1-yl)ethyl)thiourea (7). Following the general method A and starting from 3-chlorophenyl isothiocyanate (77 µl, 0.5 mmol) and 1-(2-aminoethyl)piperidine (80 µl, 0.5 mmol), thiourea **7** was obtained as an off-white solid (191 mg, 96% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.82 (br s, 1H) , 7.75 (br s, 1H), 7.71 (s, 1H), 7.33-7.30 (m, 2H), 7.15-7.12 (m, 1H), 3.59-3.51 (m, 2H), 2.45 (t, J = 6.2 Hz, 2H), 2.40-2.33 (m, 4H), 1.53-1.48 (m, 4H), 1.41-1.36 (m, 2H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 179.8, 141.0, 132.7, 130.2, 123.4, 121.7, 120.7, 56.4, 53.8, 41.1, 25.5, 24.0. HRMS calcd for $[C_{14}H_{20}ClN_3S+H]^+$ 298.1139, found 298.1136.

1-(3-Chloro-4-methoxyphenyl)-3-(2-morpholinoethyl)thiourea (8). Method C. 3-Chloro-4-methoxyaniline (514 mg, 3.26 mmol, 1.0 eq.) was added to a solution of thiophosgene (0.25 ml, 3.3 mmol, 1.0 eq.) in 3 ml of $CHCl_3$ at 0 °C. Et_3N (0.91 ml, 6.5 mmol, 2.0 eq.) was added, and the reaction was stirred for 5 min. 2-(4-Morpholino)ethylamine (0.43 ml, 3.3 mmol, 1.0 eq.) was added, and the reaction was stirred at 0 °C for 2 h. The reaction mixture was diluted with EtOAc and extracted four times with HCl (aq., 1 M). The combined aqueous phases were made basic by the addition of NaOH (aq., 2 M), extracted three times with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated to give a brown oil. The oil was dissolved in 12 ml of hot toluene/heptane 1:1 to give thiourea **8** as off-white microcrystals after cooling (672 mg, 2.04 mmol, 63% yield). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.57 (br s, 1H), 7.54 (br s, 1H), 7.53 (d, J = 2.4 Hz, 1H), 7.23 (dd, J = 8.8, 2.4 Hz, 1H), 7.11 (d, J = 8.8 Hz, 1H), 3.84 (s, 3H), 3.59-3.53 (m, 6H), 2.47 (t, J = 6.2 Hz, 2H), 2.43-2.34 (m, 4H); ^{13}C NMR (150 MHz, DMSO-*d6*) δ 180.3, 151.6, 132.4, 125.4, 123.7, 120.5, 112.7, 66.2, 56.4 (br), 56.2, 53.1, 40.7. HRMS calcd for $[C_{14}H_{20}ClN_3O_2S+H]^+$ 330.1038, found 330.1033.

1-(4-Methoxyphenyl)-3-(2-morpholinoethyl)thiourea (9). Modified method A. To 4-methoxyphenyl isothiocyanate (70 µl, 0.51 mmol, 1.0 eq.) in 5 ml of dried CH_2Cl_2, 1-(2-aminoethyl)morpholine (67 µl, 0.51 mmol, 1.0 eq.) was added dropwise at rt while stirring. The reaction vial was purged with N_2, sealed, and stirred at 70 °C for 15 minutes in a microwave reactor. After attaining rt, the reaction mixture was washed with sat $NaHCO_3$ (aq.) followed by brine, and the aqueous layers were extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The crude product was dissolved in CH_2Cl_2 and precipitated by the addition of heptane, and the formed crystals were washed with a small amount of heptane to give thiourea **9** (131 mg, 88% yield). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.47 (br s, 1H), 7.33 (br s, 1H), 7.23 (d, J = 8.8 Hz, 2H), 6.91 (d, J = 8.8 Hz, 2H), 3.74 (s, 3H), 3.58-3.50 (m, 6H), 2.45 (t, J = 6.3 Hz, 2H), 2.42-2.33 (m, 4H); ^{13}C

NMR (150 MHz, DMSO-*d6*) δ 180.3, 156.6, 131.5, 125.7, 114.1, 66.2, 56.4, 55.3, 53.0, 40.7. HRMS calcd for $[C_{14}H_{21}N_3O_2S+H]^+$ 296.1427, found 296.1419.

1-(3-Chlorophenyl)-3-(2-morpholinoethyl)thiourea (10). Following the general method A and starting from 3-chlorophenyl isothiocyanate (77 µl, 0.6 mmol) and 1-(2-aminoethyl)morpholine (77 µl, 0.6 mmol), thiourea **10** was obtained as an off-white solid (173 mg, 96% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.82 (br s, 1H), 7.81 (br s, 1H), 7.71 (s, 1H), 7.36-7.13 (m, 2H), 7.15-7.12 (m, 1H), 3.61-3.58 (m, 6H), 2.50 (m, 2H), 2.45-2.38 (m, 4H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 178.9, 140.9, 132.8, 130.2, 123.5, 121.7, 120.7, 66.2, 56.3, 53.1, 40.7. HRMS calcd for $[C_{13}H_{18}ClN_3OS+H]^+$ 300.0932, found 300.0926.

1-(4-Chloro-3-methoxyphenyl)-3-(2-morpholinoethyl)thiourea (11). Method B. Thiocarbonyldiimidazole (81 mg, 0.46 mmol, 1.0 eq.) and 4-chloro-3-methoxyaniline (72 mg, 0.46 mmol, 1.0 eq.) were stirred in 2 ml of dried MeCN in a thick-walled vial. The vial was sealed and heated to 100 °C for 10 min in a microwave reactor. 2-(4-Morpholino)ethylamine (0.06 ml, 0.5 mmol, 1 eq.) was added, and the vessel was heated to 100 °C for 10 min. The solution was concentrated in vacuo, and the resulting solid was taken up in EtOAc and washed three times with $NaHCO_3$ (aq., sat.). The organic phase was dried over Na_2SO_4, filtered, and concentrated. The crude product was recrystallized from toluene, but a remaining contaminant required further purification by dissolution in CH_2Cl_2 (3 ml) and precipitation of the product by addition of heptane (10 ml). After washing with heptane, thiourea **11** was obtained as white microcrystals (74 mg, 0.22 mmol, 49% yield). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 9.77 (br s, 1H), 7.70 (br s, 1H), 7.37 (d, *J* = 2.0 Hz, 1H), 7.34 (d, *J* = 8.5 Hz, 1H), 6.99 (dd, *J* = 8.5, 2.0 Hz, 1H), 3.82 (s, 3H), 3.62-3.54 (m, 6H), 2.49 (t, *J* = 6.3 Hz, 2H), 2.44-2.35 (m, 4H); ^{13}C NMR (150 MHz, DMSO-*d6*) δ 179.9, 154.3, 139.3, 129.5, 115.8, 115.3, 107.5, 66.2, 56.4, 56.0, 53.2, 40.7. HRMS calcd for $[C_{14}H_{20}ClN_3O_2S+H]^+$ 330.1038, found 330.1033.

1-(3-Chloro-4-methoxyphenyl)-3-(2-(dimethylamino)ethyl)thiourea (12). Method B. Thiocarbonyldiimidazole (113 mg, 0.63 mmol, 1.0 eq.) and 3-chloro-4-methoxyaniline (100 mg, 0.63 mmol, 1.0 eq.) were stirred in 2 ml of dried MeCN in a thick-walled vial. The vial was sealed and heated to 100 °C for 10 min in a microwave reactor. *N*,*N*-Dimethylethylenediamine (0.07 ml, 0.6 mmol, 1 eq.) was added, and the vessel was heated to 100 °C for 10 min. The solution was concentrated in vacuo, and the resulting solid was taken up in EtOAc and washed three times with $NaHCO_3$ (aq., sat.). The organic phase was dried over Na_2SO_4, filtered, and concentrated. The crude product was recrystallized from a mixture of 1.5 ml of CH_2Cl_2 and 12 ml of heptane to give thiourea **12** as off-white flakes (134 mg, 0.47 mmol, 73% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.57 (br s, 1H), 7.59 (d, *J* = 2.6 Hz, 1H), 7.57 (br s, 1H), 7.23 (dd, *J* = 8.8, 2.6 Hz, 1H), 7.09 (d, *J* = 8.8 Hz, 1H), 3.83 (s, 3H), 3.59-3.45 (m, 2H), 2.41 (t, *J* = 6.2 Hz, 2H), 2.18 (s, 6H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 180.5, 151.4, 132.8, 125.2, 123.4, 120.3, 112.6, 57.3, 56.2, 45.0, 41.7. HRMS calcd for $[C_{12}H_{18}ClN_3OS+H]^+$ 288.0932, found 288.0929.

1-(2-(Dimethylamino)ethyl)-3-(4-methoxyphenyl)thiourea (13). Following the general method A and starting from 4-metoxyphenyl isothiocyanate (69 µl, 0.5 mmol) and *N*,*N*-dimethylethylenediamine (55 µl, 0.5 mmol), thiourea **13** was obtained as an off-white solid (110 mg, 87% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.46 (br s, 1H), 7.37 (br s, 1H), 7.25 (d, *J* = 8.9 Hz, 2H), 6.88 (d, *J* = 8.9 Hz, 2H), 3.73 (s, 3H), 3.55-3.48 (m, 2H), 2.39 (t, *J* = 6.2 Hz, 2H), 2.16, (s, 6H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 180.6, 156.4, 131.9, 125.5, 113.9, 57.5, 55.3, 45.1, 41.8. HRMS calcd for $[C_{12}H_{19}N_3OS+H]^+$ 254.1322, found 254.1315.

1-(3-Chlorophenyl)-3-(2-(dimethylamino)ethyl)thiourea (14). Following the general method A and starting from 3-chlorophenyl isothiocyanate (77 µl, 0.6 mmol) and *N*,*N*-dimethylethylenediamine (64 µl, 0.6 mmol), thiourea **14** was obtained as an off-white solid (130 mg, 86% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.84 (br s, 1H), 7.81 (s, 1H), 7.80 (br s, 1H), 7.33-7.31 (m, 2H), 7.12-7.10 (m, 1H), 3.59-3.51 (m, 2H), 2.44 (t, *J* = 6.1 Hz, 2H),

2.19 (s, 6H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 180.0, 141.2, 132.6, 130.1, 123.2, 121.5, 120.5, 57.0, 44.9, 41.6. HRMS calcd for $[C_{11}H_{16}ClN_3S+H]^+$ 258.0826, found 258.0822.

1-(2-(Piperidin-1-yl)ethyl)-3-(4-(trifluoromethyl)phenyl)thiourea (15). Method B. Thiocarbonyldiimidazole (100 mg, 0.56 mmol, 1.0 eq.) and 4-(trifluoromethyl)aniline (0.07 ml, 0.6 mmol, 1 eq.) were stirred in 2 ml of dried MeCN in a thick-walled vial. The vial was sealed and heated to 100 °C for 10 min in a microwave reactor. 1-(2-Aminoethyl)piperidine (0.08 ml, 0.6 mmol, 1 eq.) was added, and the vessel was heated to 100 °C for 10 min. The solution was concentrated in vacuo, and the resulting solid was taken up in EtOAc and washed three times with $NaHCO_3$ (aq., sat.). The organic phase was dried over Na_2SO_4, filtered, and concentrated to give a dark solid. Careful recrystallization from heptane gave thiourea **15** as a pale yellow powder (61 mg, 0.18 mmol, 33% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 10.02 (br s, 1H), 7.88 (br s, 1H), 7.74 (d, *J* = 8.5 Hz, 2H), 7.64 (d, *J* = 8.5, 2H), 3.64-3.50 (m, 2H), 2.46 (t, *J* = 6.2 Hz, 2H), 2.43-2.28 (m, 4H), 1.57-1.45 (m, 4H), 1.44-1.35 (m, 2H); ^{13}C NMR (150 MHz, DMSO-*d6*) δ 179.9, 143.4, 125.7, 124.4 (q, *J* = 271 Hz), 123.2 (q, *J* = 32 Hz), 121.6, 56.4, 53.9, 41.2, 25.5, 24.0. ^{19}F NMR (376 MHz, DMSO-*d6*) δ -60.81. HRMS calcd for $[C_{15}H_{20}F_3N_3S+H]^+$ 332.1403, found 332.1400.

1-(2-(Morpholin-4-yl)ethyl)-3-(4-sulfamoylphenyl)thiourea (16). Modified method A. 4-(2-Aminoethyl)morpholine (0.05 ml, 0.4 mmol, 1.0 eq.) was added to 4-isothiocyanatobenzenesulfonamide[40] (86 mg, 0.40 mmol, 1.0 eq.) in 3 ml of CH_2Cl_2 in a thick-walled vial. The vial was sealed and heated to 70 °C for 10 min in a microwave reactor. The white suspension was concentrated and recrystallized from 7 ml of EtOH to give thiourea **16** as white crystals (112 mg, 0.33 mmol, 81% yield). ^{1}H NMR (400 MHz, DMSO-*d6*, 333 K) δ 9.86 (br s, 1H), 7.82 (br s, 1H), 7.75 (d, *J* = 8.8 Hz, 2H), 7.68 (d, *J* = 8.8 Hz, 2H), 7.14 (br s, 2H), 3.66-3.56 (m, 6H), 2.53 (t, *J* = 6.3 Hz, 2H), 2.46-2.40 (m, 4H); ^{13}C NMR (100 MHz, DMSO-*d6*, 333 K) δ 180.1, 142.5, 138.4, 126.0, 121.3, 66.0, 56.1, 52.9, 40.6. HRMS calcd for $[C_{13}H_{20}N_4O_3S_2+H]^+$ 345.1050, found 345.1046.

1-(4-Chlorophenyl)-3-(2-morpholinoethyl)thiourea (17). Method B. Thiocarbonyldiimidazole (100 mg, 0.56 mmol, 1.0 eq.) and 4-chloroaniline (72 mg, 0.56 mmol, 1.0 eq.) were stirred in 2 ml of dried MeCN in a thick-walled vial. The vial was sealed and heated to 100 °C for 10 min in a microwave reactor. 2-(4-Morpholino)ethylamine (0.07 ml, 0.6 mmol, 1 eq.) was added, and the vessel was heated to 100 °C for 10 min. The solution was concentrated in vacuo, and the resulting solid was taken up in EtOAc and washed three times with $NaHCO_3$ (aq., sat.). The organic phase was dried over Na_2SO_4, filtered, and concentrated. The obtained crude product was recrystallized from toluene to give thiourea **17** as off-white small flakes (130 mg, 0.43 mmol, 77% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.75 (br s, 1H), 7.69 (br s, 1H), 7.48 (d, *J* = 8.7 Hz, 2H), 7.36 (d, *J* = 8.7 Hz, 2H), 3.64-3.51 (m, 6H), 2.49-2.44 (m, 2H), 2.44-2.35 (m, 4H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 180.1, 138.3, 128.5, 127.8, 124.3, 66.2, 56.4, 53.1, 40.7. HRMS calcd for $[C_{13}H_{18}ClN_3OS+H]^+$ 300.0932, found 300.0924.

1-(2-(Dimethylamino)ethyl)-3-(4-sulfamoylphenyl)thiourea (18). Modified method A. *N*,*N*-dimethylethylenediamine (0.06 ml, 0.55 mmol, 1.1 eq.) was added to 4-isothiocyanatobenzenesulfonamide[40] (106 mg, 0.49 mmol, 1.0 eq.) in 3 ml of CH_2Cl_2 in a thick-walled vial. The vial was sealed and heated to 70 °C for 10 min in a microwave reactor. The mixture was allowed to attain rt and concentrated. The resulting crude mixture was dissolved in dioxane (1 ml) and the product was precipitated by addition of toluene (15ml). The solvent was decanted and the resulting solid was twice treated with toluene and sonicated to give thiourea **18** as white solid (148 mg, 0.49 mmol, 99% yield). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 10.05 (br s, 1H), 7.92 (br s, 1H), 7.76-7.67 (m, 4H), 7.25 (br s, 2H), 3.65-3.47 (m, 2H), 2.47 (t, *J* = 5.9 Hz, 2H), 2.22 (s, 6H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 180.1, 142.8, 138.3, 126.2, 121.2, 57.0, 44.9, 41.5. HRMS calcd for $[C_{11}H_{18}N_4O_2S_2+H]^+$ 303.0944, found 303.0942.

1-(2-Chlorophenyl)-3-(2-(dimethylamino)ethyl)thiourea sulfate (19). Method C. 2-Chloroaniline (0.14 ml, 1.3 mmol, 1.0 eq.) was added dropwise to a solution of thiophosgene (0.10 ml, 1.3 mmol, 1.0 eq.) in 1.5 ml of $CHCl_3$

at −10 °C. To the formed yellow-white precipitate was added Et_3N (0.36 ml, 2.6 mmol, 2.0 eq.), and the vessel contents cleared. *N*,*N*-Dimethylethylenediamine (0.14 ml, 1.3 mmol, 1.0 eq.) was added dropwise, and the reaction was allowed to reach r.t. over 2 h. The dark red reaction mixture was diluted with EtOAc and extracted three times with HCl (aq., 1 M). The obtained aqueous phase was made basic by the addition of NaOH (aq., 2 M) and extracted three times with CH_2Cl_2. The organic phase was dried over Na_2SO_4, filtered and concentrated to give a brown oil. The oil was dissolved in 10 ml of EtOH, and treated with 48 mg of H_2SO_4 (ca. 0.5 eq.) dissolved in 1.2 ml of EtOH. The product precipitated slowly as transparent needles, which were triturated with cold EtOH to give thiourea **19** after removing residual solvent in vacuo (209 mg, 0.68 mmol, 52% yield). ^{1}H NMR (600 MHz, DMSO-*d6*, 323 K) δ 9.40 (br s, 1H), 7.97-7.90 (m, 1H), 7.62 (d, *J* = 7.8 Hz, 1H), 7.49 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.32 (dt, *J* = 1.3, 7.6 Hz, 1H), 7.24 (dt, *J* = 1.5, 7.7 Hz, 1H), 3.76-3.67 (m, 2H), 3.26 (v br s, 1H), 2.92-2.86 (m, 2H), 2.54 (s, 6H); ^{13}C NMR (150 MHz, DMSO-*d6*, 323 K) δ 181.7, 135.9, 129.3, 129.2, 127.1, 127.0, 56.3, 43.7, 40.4. We were unable to resolve all 6 aromatic carbon peaks in either DMSO-*d6* (sulfate) or $CDCl_3$ (freebase). HRMS calcd for $[C_{11}H_{16}ClN_3S+H]^+$ 258.0826, found 258.0825.

1-(2-Methoxyphenyl)-3-(2-(piperidin-1-yl)ethyl)thiourea hydrochloride (20). Method B. Thiocarbonyldiimidazole (103 mg, 0.58 mmol, 1.0 eq.) and 2-methoxyaniline (0.07 ml, 0.6 mmol, 1 eq.) were stirred in 2 ml of dried MeCN in a thick-walled vial. The vial was sealed and heated to 100 °C for 10 min in a microwave reactor. 1-(2-Aminoethyl)piperidine (0.08 ml, 0.6 mmol, 1 eq.) was added, and the vessel was heated to 100 °C for 10 min. The solution was concentrated in vacuo, and the resulting solid was taken up in EtOAc and washed three times with $NaHCO_3$ (aq., sat.). The organic phase was dried over Na_2SO_4, filtered, and concentrated to give the crude product as an oil. The oil was dissolved in EtOAc, and the corresponding hydrochloride salt was precipitated by the addition of HCl in Et_2O (1.0 M, 1.5 ml). Concentration in vacuo and recrystallization from 10 ml of EtOH gave thiourea **20** as red crystals (111 mg, 0.34 mmol, 58% yield). ^{1}H NMR (600 MHz, DMSO-*d6*) δ 10.35 (br s, 1H), 9.22 (s, 1H), 8.17 (br s, 1H), 7.63 (br d, *J* = 7.0 Hz, 1H), 7.18 (t, *J* = 7.6 Hz, 1H), 7.05 (d, *J* = 8.2 Hz, 1H), 6.92 (t, *J* = 7.6 Hz, 1H), 3.92-3.85 (m, 2H), 3.79 (s, 3H), 3.46-3.39 (m, 2H), 3.23-3.16 (m, 2H), 2.99-2.88 (m, 2H), 1.83-1.73 (m, 4H), 1.72-1.65 (m, 1H), 1.44-1.33 (m, 1H); ^{13}C NMR (150 MHz, DMSO-*d6*) δ 181.0, 152.5, 126.9, 126.4, 126.3, 120.0, 111.8, 55.6, 54.7, 52.3, 38.4, 22.4, 21.2. HRMS calcd for $[C_{15}H_{23}N_3OS+H]^+$ 294.1635, found 294.1636.

1-(2-Methoxyphenyl)-3-(2-morpholinoethyl)thiourea (21). Method B. Thiocarbonyldiimidazole (100 mg, 0.56 mmol, 1.0 eq.) and 2-methoxyaniline (0.06 ml, 0.6 mmol, 1 eq.) were stirred in 2 ml of dried MeCN in a thick-walled vial. The vial was sealed and heated to 100 °C for 10 min in a microwave reactor. 2-(4-Morpholino)ethylamine (0.07 ml, 0.6 mmol, 1 eq.) was added, and the vessel was heated to 100 °C for 10 min. The vessel contents were taken up in EtOAc and washed three times with $NaHCO_3$ (aq., sat.). The organic phase was extracted three times with HCl (aq., 1 M); the obtained aqueous phase were made basic by the addition of NaOH (aq., 2 M) and extracted three times with EtOAc. The organic phase was dried over Na_2SO_4, filtered and concentrated to give a clear oil, which was flash chromatographed in heptane/EtOAc 2:8 to 1:9 to give thiourea **21** as a transparent, thick oil (123 mg, 0.42 mmol, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (br s, 1H), 7.34-7.21 (m, 1H), 7.15 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.12-7.05 (m, 1H), 6.94-6.88 (m, 2H), 3.77 (s, 3H), 3.67-3.56 (m, 2H), 3.52-3.41 (m, 4H), 2.47 (t, *J* = 5.9 Hz, 2H), 2.40-2.27 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.8, 152.2, 127.2, 125.6, 124.6, 120.7, 111.8, 66.7, 55.8, 55.6, 52.8, 41.2. HRMS calcd for $[C_{14}H_{21}N_3O_2S+H]^+$ 296.1427, found 296.1441.

5.7. Protein expression

The expression of recombinant AChE1 enzymes from the mosquitoes *An. gambiae* [21], the G122S mutant of *Ag*AChE1 [21], and *Ae. aegypti* [21], as well as recombinant *h*AChE [41] was performed as described previously.

5.8. IC_{50} determinations

In order to study SAR and SSR on thiourea scaffold-based compounds, dose-response experiments were performed with a small library of analogues of this compound class on the four target proteins (*Ag/Aa*AChE1, *h*AChE and G122S-*Ag*AChE1). The recombinant enzymes used in this study have previously been probed with commercial reference compounds, both covalent and non-covalent inhibitors [21]. Stock solutions of the synthesized compounds were prepared in DMSO at 100 mM, and working dilutions were prepared in either sodium phosphate buffer (0.1 M, pH 7.4) or MilliQ water, depending on solubility preferences. Compound solutions of eight different concentrations up to a maximum of 1 mM were used. The activity measurements were performed using secreted non-purified proteins in growth medium, and enzymatic activity was measured using the Ellman assay [42] adapted to a 96-well format. Automatic handling of liquids for assay plate setup was performed using a QIAgility robotic benchtop instrument (Qiagen). Assays were performed at 30 °C in a final volume of 200 µL of 0.1 M phosphate buffer (pH 7.4) containing 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 1 mM acetylthiocholine iodide. The enzymatic reaction was measured by monitoring changes in the absorbance of individual wells at 412 nm over 60 s in a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices). The average slope determined for eight positive (uninhibited) controls on each plate was taken to represent 100% activity, and the activity observed in the sample wells were quantified in relation to this value. IC_{50} values were calculated using non-linear regression (curve fitting) in GraphPad Prism [43], and the log [inhibitor] vs. response variable slope equation was fitted using three parameters. IC_{50} values were determined using 2-3 independent replicates. The lower catalytic activity in the dose-response experiments of the G122S mutant of the *Ag*AChE1 enzyme led to a reduced signal to noise ratio compared to wild type *Ag*AChE1 (*cf.* CI in Tables 1-3). The intrinsic reactivities between 5,5'-dithiobis(2-nitrobenzoic acid) and compounds **3** and **13** were tested in phosphate buffer (pH 7.4), and was found to be negligible on the timescale of the assay.

5.9. Inhibitory effect of thioureas on mosquito extract

The inhibitory effect of thiourea-based compounds on mosquito extract was investigated using a method modified from [44]. 30 adult *Ae. aegypti* female mosquitoes stored at -20°C were sorted two per tube, the heads separate from the bodies. The samples were homogenized by manual grinding in 100 µL phosphate buffer pH 7.4 containing 1% Triton X-100. The grinder was rinsed with 300 µL phosphate buffer and the samples were subsequently vortexed and centrifuged at 10 000 rpm for 3 min at 4°C. The supernatant from all samples were pooled together, still heads separate from bodies. 100 µL of pooled sample were used per measurement and the Ellman assay described before was used for IC_{50} determinations of head- and body-samples.

5.10. In vivo experiments with *An. gambiae* and *Ae. aegypti*

An. gambiae s.s. Kisumu strain and *Ae. aegypti* Mombasa strain, both from Kenya, were used to test the insecticial activities of the compounds. Mosquito rearing was carried out in an insectary maintained at 27-28 °C at ca. 80% humidity, on a 12/12 h light/darkness cycle, and maintained at optimal larval concentrations to avoid possible effects of competition. Mosquito larvae were reared in de-chlorinated tap water, and were fed on finely ground Sera Vipan staple diet™ (Sera, Germany), while adults were offered a fresh 10% (w/v) glucose solution meal daily, and were fed on hamster (*Mesocricetus auratus*) as a source of blood meals when egg production was desired. Insecticidal activity tests of the compounds were carried out following World Health Organization guidelines for testing adulticides and larvicides [45, 46].

For the adult mosquito tests, non-blood fed, five day old female mosquitoes were used, and testing was performed in batches of five mosquitoes each. Each batch of mosquitoes was placed in a 500 ml paper cup and anesthetized by placing the cup in a -20 °C freezer for 3 minutes. Thereafter, the mosquitoes were gently

poured onto a plate refrigerated at -20 °C overlaid with a paper towel, and the compound solution (acetone, 0.1 µl) was deposited on the upper part of the pronotum using a micropipetter. As a negative control, 0.1 µl of acetone was used, while for the positive control, 6.25 ng of propoxur (2-isopropoxyphenyl-*N*-methylcarbamate) in 0.1 µl of acetone was used [47]. After the topical application, the mosquitoes were returned to the insectary, where they were supplied with a glucose meal and maintained under standard conditions. The mortality rates were recorded after 24 h. The mortality rates were adjusted using Abbott's formula[36] in cases where mortality in the control was over 5%. No experiment had a >20% mortality in controls.

Larval tests were conducted in the following manner. For each tested compound and concentration, 25 larvae at third instar were introduced into a cup containing compound dissolved in 100 ml of de-chlorinated water, and maintained in the insectary under standard conditions. Negative control experiments comprised of larvae maintained in plain de-chlorinated water while the positive controls were conducted with propoxur at 25 µM. Mortality rates were recorded after 24 h.

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Keywords: acetylcholinesterase 1 • vector control • *Anopheles gambiae* • *Aedes aegypti* • insecticides • thiourea

Additional information

Supporting information includes ^{1}H and ^{13}C NMR spectra of synthesized compounds, dose-response curves for IC_{50} determination of recombinant *Ag*AChE1, *Aa*AChE1, *h*AChE and G122S-*Ag*AChE1 and details about the mosquito experiments.

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Highlights

- 21 aryl-ethyleneamino substituted thioureas were synthesized and evaluated
- Compounds inhibit mosquito AChE1 with IC_{50} values from 0.1 µM
- Several compounds show selectivity for mosquito AChE1 over human AChE
- Insecticidal activity was demonstrated on *Aedes aegypti* and *Anopheles gambiae*