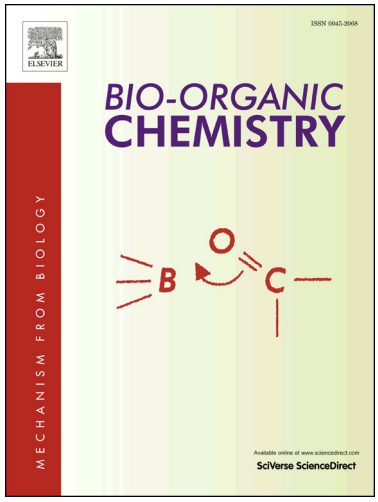

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Synthesis and *in vitro* evaluation of novel rhodanine derivatives as potential cholinesterase inhibitors

Martin Krátký [a,*], Šárka Štěpánková [b], Katarína Vorčáková [b], and Jarmila Vinšová [a]

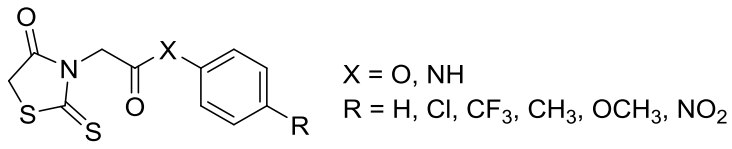
[a] Department of Inorganic and Organic Chemistry, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 532 10 Pardubice, Czech Republic

*Corresponding author. Heyrovského 1203, 500 05 Hradec Králové, Czech Republic. E-mail address: martin.kratky@faf.cuni.cz, tel.: +420-495067343, fax: +420-495067166.

Abstract

Based on a broad spectrum of biological activities of rhodanines, we synthesized aromatic amides and esters of 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid (rhodanine-3-acetic acid) *via* carbodiimide- or PCl_3-mediated coupling. Both esters and amides were investigated for their *in vitro* inhibitory potency and selectivity against acetylcholinesterase (AChE) from electric eel and butyrylcholinesterase (BChE) from equine serum using Ellman's spectrophotometric method. The derivatives exhibited mostly a moderate activity against both cholinesterases. IC_{50} values for AChE were in a closer concentration range of 24.05-86.85 µM when compared to BChE inhibition (7.92-227.19 µM). The esters caused the more efficient inhibition of AChE than amides and parent acid. The esterification and amidation of the rhodanine-3-acetic acid increased inhibition of BChE, even up to 26 times. Derivatives of 4-nitroaniline/phenol showed the activity superior to other substituents (H, Cl, CH_3, OCH_3, CF_3). Rhodanines produced a balanced inhibition of both cholinesterases. Seven derivatives produced the more potent inhibition of AChE than rivastigmine, a clinically used drug; additional three compounds were comparable. Two amides exceeded inhibitory potency of rivastigmine towards BChE. Importantly, this is the first evidence that rhodanine-based compounds are able to inhibit BChE.

Graphical abstract



IC_{50} values for acetylcholinesterase from 24.05 µmol/L
IC_{50} values for butyrylcholinesterase from 7.92 µmol/L

Keywords

acetylcholinesterase; butyrylcholinesterase; enzyme inhibition; 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid; rhodanine[*]

[*]Abbreviations: ACh: acetylcholine; AD: Alzheimer´s disease; AChE: acetylcholinesterase; BBB: blood brain barrier; BChE: butyrylcholinesterase; ChE: cholinesterases; CNS: central nervous system.

1. Introduction

Rhodanine (2-thioxothiazolidin-4-one) has become an interesting important heterocyclic moiety in medicinal chemistry owing to its wide spectrum of biological activities through different mechanisms of action. Due to various possibilities of chemical modification, rhodanine-based compounds were considered as a privileged scaffold in drug discovery [1].

Rhodanines have been reported as, e.g., potential antibacterial [2,3,4,5], antifungal [2,6,7], antiviral [8], anticancer [9] and anti-inflammatory [10] agents. They are considered to be useful for the treatment and prevention of diabetes-related complications [11]. Also many enzymes have been identified as their potential molecular targets. Additionally, they can potentially be used in the treatment of obesity, neurologic and psychiatric disorders, cystic fibrosis etc. [1]. Generally, rhodanines were found to be nonmutagenic [12].

However, they have gained a reputation as promiscuous, pan assay interference compounds (PAINS) and aggregators that can interact non-specifically with target proteins as well as undergo Michael 1,4-conjugative addition with various nucleophiles in the case of 5-arylmethylidenerhodanines [13]. The 5-benzylidene substitution was also reported to complicate the investigation in cell-based assays as well as *in vivo* because they can react with glutathione and other compounds with free thiol groups within cells [1,13].

The prevalence of dementias has been increased globally as well as health-care expenses for their treatment. Alzheimer´s disease (AD) represents the most frequent cause characterized by a progressive deterioration in cognition, in particular the memory domain, function and behaviour. The incidence rate for dementia increases exponentially with age [14].

The exact cause of the AD is still uncertain, but several hypotheses have been proposed. Based on cholinergic hypothesis, deficiency of acetylcholine (ACh) in brain was observed either due to decreased production or amplified acetylcholinesterase (AChE) activity. This decreased level of the neurotransmitter causes impairment of the cholinergic neurotransmission and the cholinergic augmentation will improve the symptoms of AD [15]. Principal physiological function of AChE (EC 3.1.1.7) is the hydrolysis of ACh, resulting in the termination of the nerve impulse. Also butyrylcholinesterase (also called pseudocholinesterase or plasma cholinesterase; BChE; EC 3.1.1.8) terminates the action of ACh [15,16].

It is necessary to identify novel drugs that prevent, delay the onset, slow the progression, or improve the symptoms of AD. Today, only four cholinesterase inhibitors (tacrine, donepezil, rivastigmine, galantamine) and an *N*-methyl-D-aspartate receptor antagonist (memantine) are approved. No new treatments have been marketed for the treatment of AD since 2003. Unfortunately, many failures have occurred [17]. Several rhodanine derivatives have been reported as potentially useful agents for the treatment of Alzheimer´s disease: inhibitors of glycogen synthase kinase-3 [18,19], tau aggregation [20,21] or AChE [10] inhibitors.

2-(4-Oxo-2-thioxothiazolidin-3-yl)acetic acid (rhodanine-3-acetic acid) represents one of the frequent thiazolidine/rhodanine-based fragments. However, a majority of reported rhodanine-3-acetic acid derivatives have been based on Knoevenagel reaction with aromatic aldehydes to form 5-arylidenerhodanines. The compounds with substituted carboxyl functional group are rarer.

As a part of our ongoing screening and search for novel cholinesterases inhibitors [22,23,24] we designed and synthesized a series of aromatic rhodanine-3-acetic acid esters and amides. To avoid possible non-specific reactivity as Michael acceptors, rhodanine-3-acetic acid was not reacted with aldehydes and only carboxyl group was modified. To the best of our knowledge, rhodanine-3-acetic

acid (2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid) and its derivatives with unsubstituted carbon 5 in the thiazolidine ring have not been reported in the literature to date as inhibitors of neither AChE nor BChE.

An ideal potential cholinesterase inhibitor should combine a powerful enzyme inhibition (IC_{50} values in submicromolar range), a lack of cytotoxicity for mammalian cells and optimal physicochemical properties for drug-likeness and crossing the blood brain barrier (BBB). From a theoretical point of view, dual inhibition of both cholinesterases may be beneficial.

2. Results and Discussion

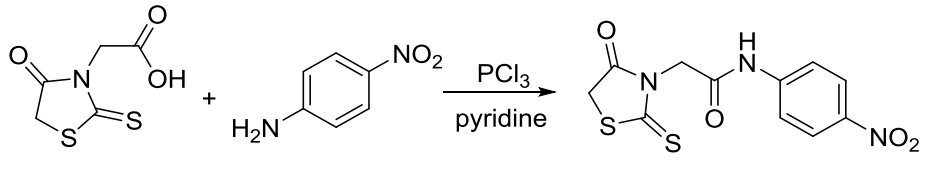
2.1 Chemistry

We have chosen several substituents with different electronic effects as a substitution pattern for aniline and phenol used for the derivatization of the 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid **1** (Table 1): H (no effect), Cl (-I and +M effects), CF_3 (-I effect), CH_3 (+I effect), OCH_3 (+M and -I effects) and NO_2 (-I and -M effects).

Amides of rhodanine-3-acetic acid **2** were obtained by two synthetic procedures. Method A, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC)-mediated coupling in the presence of 1-hydroxybenzotriazole (HOBt; Scheme 1) was successful for five amides providing yields within the range of 76-95%. Reaction of 4-(trifluoromethyl)aniline provides the lowest but still acceptable yield (76 %) due to strong electron-withdrawing properties of the CF_3 moiety. However, this method failed in the case of 4-nitroaniline. Other additional attempts with different reaction conditions, changing carbodiimides, additives, using 1,1´-carbonyldiimidazole and mixed anhydrides methods led also to an unsatisfactory outcome. Then, we applied PCl_3-mediated reaction in dry pyridine (Method B; Scheme 2; yield of **2f** was 78 %) according to Rijkers et al. [25], which is useful for very weak nucleophiles like 4-nitroaniline.

1 **2**

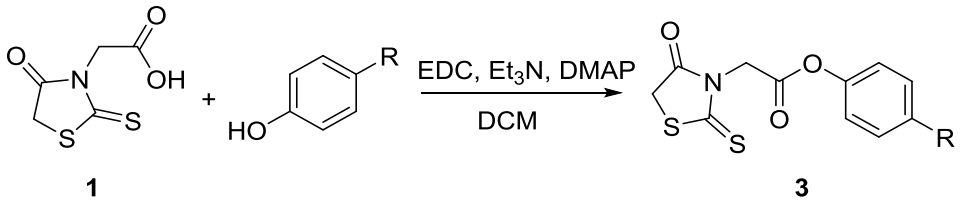
Scheme 1. Synthesis of amides **2** (EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; HOBt = 1-hydroxybenzotriazole; DCM = dichloromethane; EtOAc = ethyl acetate; R = H, Cl, CF_3, CH_3, OCH_3, NO_2).



1 **2f**

Scheme 2. Synthesis of 4-nitroanilide **2f**

Esters **3** were obtained by Steglich-type esterification employing EDC and DMAP (Scheme 3). The general yields ranged from 66 % (4-(trifluoromethyl)aniline derivative **3c**) up to 79 %.



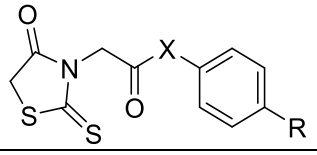
Scheme 3. Synthesis of esters **3** (EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; DMAP = 4-(dimethylamino)pyridine; Et_3N = triethylamine; DCM = dichloromethane; R = H, Cl, CF_3, CH_3, OCH_3, NO_2).

Compounds were characterized by melting points, IR and NMR spectra; their purity was checked by thin-layer chromatography and elemental analysis. ^{1}H NMR spectra of the amides **2** contained singlets attributed to the amidic N-H proton in the ranges of 10.17-10.48 ppm (**2b**, **2d**, **2e**) and 8.78-8.82 ppm in the case of aniline derivatives with electron-withdrawing substituents (**2c**, **2f**). Methylene group connecting heterocyclic nitrogen (N-3) and carboxamide produced a sharp singlet in the range of 4.66-4.87 ppm. The singlets of thiazolidine -CH_2- hydrogens were observed at 4.14-4.40 ppm. ^{13}C NMR spectra contain signals of two C=O carbon atoms (amidic, heterocyclic) in the downfield region of 163.12-174.44 ppm. The chemical shift of C=S carbon is in the range of 200.89-203.57 ppm. N-3 connected with -CH_2- group showed signals around 47 ppm and thiazolidine -CH_2- around 36 ppm. In ^{1}H NMR spectra of the esters **3**, hydrogens of -CH_2- linker between N-3 and -C(=O)O- group were observed as singlets in the range of 4.93-4.99 ppm. The chemical shift of thiazolidine -CH_2- hydrogens singlets was 4.10-4.14 ppm. ^{13}C NMR spectra contain signals of two C=O carbons atoms (C=O ester, 4-oxothiazolidine) at 163.79-172.95 ppm. The signals of C=S appear in the downfield region with similar chemical shifts like those obtained for amides **2** (200.29-200.34 ppm). N-3 connected methylene linker carbon produced signals at 44.77-44.91 ppm and thiazolidine -CH_2- is present at 35.70-35.78 ppm.
In the IR spectra of esters **3**, sharp and strong bands appear at around 1751-1778 cm^{-1} (C=O ester), derivatives **2** showed amide I bands at 1654-1665 and also N-H stretch bands (3259-3300 cm^{-1}). Bands of cyclic C=O were observed in the spectra of both group compounds **2** and **3** (1731-1748 cm^{-1}).

2.2 *In vitro* inhibition of cholinesterases

Six rhodanine-based amides **2**, six esters **3** and parent acid **1** were evaluated against AChE from electric eel and BChE from equine serum using modified Ellman's method [26]. The efficacy is expressed as IC_{50} values, representing the concentration of an inhibitor required for 50% inhibition of the enzyme. The results were compared with those obtained for rivastigmine and galantamine, two clinically used ChE inhibitors with different structures and mechanism of action. Rivastigmine is an acylating pseudo-irreversible carbamate-based inhibitor affecting the function of both cholinesterases, while galantamine acts as a non-acylating competitive reversible inhibitor. Furthermore, it modulates allosterically nicotinic ACh receptors.
All of the rhodanine derivatives **1**-**3** inhibited significantly both cholinesterases with IC_{50} values from 7.92 to 227.19 μM (Table 1). Most of these results are comparable or superior to several groups of recently published ChE inhibitors [27,28].

Table 1. IC_{50} values of rhodanine derivatives **1**-**3** for acetylcholinesterase and butyrylcholinesterase



Code	X	R	IC_{50} for AChE [μM]	IC_{50} for BChE [μM]	Selectivity to AChE
1	- (free acid)		54.08 ± 1.34	205.44 ± 25.97	3.8
2a	NH	H	63.06 ± 2.86	61.06 ± 0.08	1.0
2b	NH	Cl	56.76 ± 1.47	26.57 ± 0.29	0.5
2c	NH	CF_3	86.85 ± 5.12	227.19 ± 11.56	2.6
2d	NH	CH_3	58.46 ± 0.63	77.69 ± 0.82	1.3
2e	NH	OCH_3	66.70 ± 1.77	65.37 ± 0.31	1.0
2f	NH	NO_2	30.82 ± 0.72	**7.92 ± 0.75**	0.3
3a	O	H	45.34 ± 0.62	76.80 ± 0.96	1.7
3b	O	Cl	41.53 ± 0.38	50.57 ± 0.97	1.2
3c	O	CF_3	37.79 ± 1.46	54.85 ± 1.10	1.5
3d	O	CH_3	44.52 ± 0.76	52.29 ± 0.50	1.2
3e	O	OCH_3	41.45 ± 0.04	58.03 ± 0.98	1.4
3f	O	NO_2	**24.05 ± 0.28**	51.00 ± 0.53	2.1
Rivastigmine			56.10 ± 1.41	38.40 ± 1.97	0.7
Galantamine			1.54 ± 0.02	2.77 ± 0.15	1.8

AChE and BChE inhibition is expressed as the mean ± SD (n = 3 experiments). Selectivity to AChE: IC50 for BChE/IC50 for AChE. The best results for each enzyme are shown in bold.

The IC_{50} values for AChE are within the range of 24-87 μM. 4-Nitrophenol ester **3f** was identified as the most effective inhibitor of AChE with IC_{50} of 24.05 μM, whereas 4-(trifluoromethyl)anilide **2c** led to the least potent inhibition (IC_{50} = 86.85 μM). The esters **3** caused uniformly a more efficient inhibition of AChE than corresponding amides **2**. The explanation may consist in a different interaction with target site(s) due to missing hydrogen, increased lipophilicity, slightly lower molar refractivity, or their combination (Table 2). Additionally, all of the esters **3** exhibited lower IC_{50} values than parent acid **1** (54 μM) while amides **2** with one exception (**2f**) not. With respect to the substitution of the benzene ring, 4-NO_2 group offers an inhibition superior to other substituents (H, Cl, CH_3, OCH_3, CF_3) whose activities differ among themselves only slightly. In general, the introduction of any of chlorine, methyl, methoxy, or trifluoromethyl moieties into phenyl ring did not influenced the activity significantly when compared to unsubstituted benzene.

The IC_{50} values for BChE are in comparatively broader concentration range (7.9-227.2 μM). In contrast to AChE, it is difficult to decide, members of which group are better inhibitors of BChE, because amides **2** include the best inhibitor of BChE **2f** (IC_{50} = 7.92 μM) as well as the least effective derivative **2c** (IC_{50} = 227.19 μM), which is also the least potent inhibitor of AChE. Among esters **3**, all 4-substituted aniline derivatives **3b**-**3f** showed uniform and almost identical IC_{50} values of 50.6-58.0 μM. The replacement of *para*-hydrogen (R = H) by any of remaining substituents (Cl, CF_3, CH_3, OCH_3, NO_2) decreases IC_{50} values of esters **3**, while this modification leads to an ambiguous effect in the case of amides **2**. In general, the presence of 4-nitro group (compounds **f**) produced the strongest inhibition of BChE, followed by the 4-chloro derivatives **b**. With exception of **2c**, esterification or amidation of the rhodanine-3-acetic acid **1** increased the inhibition potency for BChE, even up to 26 times (**1** vs. **2f**). These modifications influence both log*P* and MR (Table 2), or it may result in a different way of binding to the enzyme. However, among esters **3** and amides **2**, it seems that neither lipophilicity nor molar refractivity are the key factors influencing *in vitro* inhibition of BChE.

Considering the selectivity for AChE versus BChE (Table 1), most of rhodanines **1**-**3** showed a balanced inhibition of both enzymes (selectivity within the range of 0.5 to 2.0). Only amide **2f** exhibited about 3.9-fold stronger inhibition of BChE and three compounds (**1**, **2c**, **3f**) displayed significantly a more effective inhibition of AChE (selectivity higher than 2). However, no derivative exceeded the value of 10 which is considered to be borderline for the desired selectivity. Amides **2** showed a larger variability of selectivity than esters **3** (0.3-2.6 vs. 1.2-1.7, respectively). No ester **3** was more effective against BChE than against AChE; concomitantly, most of the esters (**3a**, **3b**, **3e**, and **3f**) were more selective to AChE than corresponding amides **2**. The modification of free carboxyl of the acid **1** reduced the selectivity to AChE. No direct relationship between the selectivity of enzyme inhibition and neither the lipophilicity nor molar refractivity was described.
The IC_{50} values of rhodanines **1**-**3** were compared with those obtained for galantamine and rivastigmine. All of tested compounds were significantly less active than galantamine against both enzymes. Seven derivatives (**2f**, **3a**-**3f**) showed a more potent inhibition of AChE than rivastigmine and three compounds (**1**, **2b** and **2d**) produced a comparable *in vitro* efficacy. Only amides synthesized from 4-chloro- or 4-nitroaniline (**2b**, **2f**) exceeded the inhibitory potency of rivastigmine towards BChE.
Among a wide spectrum of rhodanine derivatives, only 5-benzylidenerhodanine-3-acetamides obtained from secondary heterocyclic amines and 1-benzylpiperidin-4-amine have been reported as moderate AChE inhibitors with IC_{50} values within the range of 21 to 675 μM. Their activity towards BChE was not investigated [10]. In comparison, our rhodanines showed a more consistent inhibition of AChE (≤ 86.9 μM). Heng et al. [29] reported 5-benzylidene-2-thioxothiazolidin-4-ones without any N-3 substitution as AChE inhibitors with IC_{50} of ≥ 5.14 μM, but some molecules were inactive. However, in contrast to our derivatives **2** and **3**, all published compounds [10,29] share 5-substitution of the thiazolidine ring implying possible action as Michael acceptors. To the best of our knowledge, our report is the first evidence that rhodanine-based compounds are able to inhibit BChE so far.

2.3 Determination of octanol-1-ol/water partition coefficients, drug-likeness and polar surface area
Many drugs cross biological membranes through passive transport, which strongly depends on their lipophilicity. The necessary condition for exploitation of chosen compound *in vivo* as the brain AChE inhibitor is its ability to cross BBB. For assessment of this ability the octan-1-ol/water partition coefficient (usually expressed as $\log P_{ow}$) can be used [30,31].
Experimentally it is done by partitioning the molecule between two immiscible phases (water and octanol) and determining the P_{ow} value as the ratio of the tested compound concentration in octanol and in water. The partition coefficient serves as a quantitative descriptor of lipophilicity and is one of the key determinants of pharmacokinetic properties. According to Lipinski´s rule of five, $\log P_{ow}$ should not exceed 5 [32].
Partition coefficients of the derivatives **1**-**3** were investigated by a traditional shake-flask method and also calculated using the program CS ChemOffice Ultra version 15.0. Obtained results are shown in Table 1. Obviously, the calculated values of log*P* are in a good concordance with the experimentally determined data. Log*P* values of the esters **3** and most of the amides **2** are higher than 1 and concomitantly they do not exceed 5.
Lipinski´s rule of five deals with drug-like properties of potential oral drugs. It defines four physicochemical parameter ranges (molecular weight MWT ≤ 500, log*P* ≤ 5, number of H-bond

donors ≤ 5 and H-bond acceptors ≤ 10) that are associated with acceptable aqueous solubility and intestinal permeability required for oral bioavailability [32]. Table 2 reports both calculated and experimental log*P* values, number of H-donors (i.e., number of N-H and O-H bonds) and H-acceptors (sum of oxygen and nitrogen atoms). Molecular weights are summarized in the experimental section. Obviously, all of amides **2** and esters **3** fit this rule without any violation.
Crucially, a sufficient lipophilicity is required to allow them to cross BBB and affect central nervous system (CNS). Polar surface area (PSA) was identified as another representative parameter for CNS targeting drugs in addition to log*P* and clogD (pH 7.4; suggested preferred range of 2-4), MW (< 450) and number of hydrogen bond donors (≤ 1) [33]. PSA is defined as a sum of surfaces of polar atoms in the molecule (usually nitrogens, oxygens and hydrogen atoms bound to these heteroatoms). A PSA value of less than 60-70 $Å^2$ tends to identify active compounds [32]. According to ref. [33], suggested PSA ranges for increasing the probability of attaining improved BBB permeability to be < 90 $Å^2$, preferentially < 70. Topological PSA of the compounds **1-3** was calculated using the program CS ChemOffice Ultra version 15.0. With an exception of the most active 4-nitrophenyl-based inhibitors **2f** and **3f**, rhodanine derivatives show sufficiently low PSA values (≤ 57.61 $Å^2$).

Table 2. Physicochemical and Lipinski´s rule of five parameters of rhodanine derivatives **1-3**

Code	X	R	log*P* (calc.)	log*P* (experim.)	Number of H-bond donors	Number of H-bond acceptors	Number of violation	MR [cm^3/mol]	tPSA [$Å^2$]
1	- (free acid)		-0.24	-0.21.±.0.01	1	4	0	43.46	57.61
2a	NH	H	1.01	1.13 ± 0.08	1	4	0	70.14	49.41
2b	NH	Cl	1.57	1.49 ± 0.07	1	4	0	74.75	49.41
2c	NH	CF_3	1.93	2.01 ± 0.11	1	4	0	76.65	49.41
2d	NH	CH_3	1.5	1.45 ± 0.07	1	4	0	76.04	49.41
2e	NH	OCH_3	0.88	0.81 ± 0.02	1	5	0	77.39	58.64
2f	NH	NO_2	1.04	1.02 ± 0.05	1	7	0	ND	101.22
3a	O	H	1.69	1.75 ± 0.12	0	4	0	68.18	46.61
3b	O	Cl	2.25	2.23 ± 0.15	0	4	0	72.78	46.61
3c	O	CF_3	2.61	2.70 ± 0.13	0	4	0	74.68	46.61
3d	O	CH_3	2.17	2.05 ± 0.09	0	4	0	74.08	46.61
3e	O	OCH_3	1.56	1.60 ± 0.08	0	5	0	75.43	55.84
3f	O	NO_2	1.36	1.31 ± 0.05	0	7	0	ND	98.42

3. Conclusions

Six phenyl 2-(4-oxo-2-thioxothiazolidin-3-yl)acetates **3** and six 2-(4-oxo-2-thioxothiazolidin-3-yl)-*N*-phenylacetamides **2** were synthesized by EDC- or PCl_3-mediated coupling from rhodanine-3-acetic acid **1** in satisfactory yields. All the compounds were evaluated *in vitro* for their inhibitory potency against acetylcholinesterase from electric eel and butyrylcholinesterase from equine serum.
Rhodanine-derivatives **1-3** exhibited from weak to moderate inhibition of both cholinesterases in micromolar and low micromolar concentration range. IC_{50} values for AChE were within a closer range when compared to BChE. However, this inhibition is not selective. Several structure-activity relationships were identified. The esters **3** caused uniformly more efficient inhibition of AChE than corresponding amides **2** and the acid **1**. With respect to the substitution of the phenolic and aniline benzene ring, the presence of 4-nitro group resulted in enhanced activity when compared with other 4-substituents. The esterification or amidation of the rhodanine-3-acetic acid **1** increased strongly the inhibition potency for BChE. It seems that neither lipophilicity nor molar refractivity are the key factors influencing straightforwardly BChE inhibition. All of the tested compounds were

significantly less active than galantamine against both enzymes. Ten derivatives showed a more potent or comparable inhibition of AChE than rivastigmine and two amides exceeded its inhibitory potency against BChE.

Importantly, this is the first evidence that BChE can be inhibited by rhodanines and that rhodanines without any 5-substitution (i.e., which are not Michael acceptors) are able to affect the function of cholinesterases.

These initial set of results can stimulate a follow-up study in order to find rhodamine derivatives with submicromolar IC_{50} values. Especially nitro derivatives may be considered to be starting compounds for further structural optimization to find derivatives with substantially decreased IC_{50} values. Changing of the position of this substituent, introduction of one or more additional nitro groups, replacement of the linkage between rhodanine ring and 4-nitroaniline or 4-nitrophenol may be examples of such modifications.

4. Materials and methods

4.1 Chemistry

4.1.1 General

All of the reagents and solvents were purchased from Sigma-Aldrich (Darmstadt, Germany) or Penta Chemicals (Prague, Czech Republic), and they were used as received. The progress of the reactions and the purity of the products were monitored by thin-layer chromatography using a mixture of toluene with ethyl acetate (4:1, v/v); plates were coated with 0.2 mm Merck 60 F254 silica gel and were visualised by UV irradiation (254 nm). Melting points were determined on a Büchi Melting Point machine B-540 apparatus using open capillaries, and the reported values are uncorrected.

Elemental analysis (C, H, N) was performed on an automatic microanalyser CHNS-O CE instrument (FISONS EA 1110, Milano, Italy). Infrared spectra (ATR) were recorded on FT-IR spectrometer Nicolet 6700 FT-IR in the range of 400 to 4,000 cm^{-1}. The NMR spectra were measured in $CDCl_3$ or in DMSO-d_6 at ambient temperature on a Varian VNMR S500 instrument (500 MHz for ^1H and 125 MHz for ^{13}C; Varian Comp. Palo Alto, CA, USA). The chemical shifts, δ, are given in ppm, with respect to tetramethylsilane as an internal standard. The coupling constants (*J*) are reported in Hz.

4.1.2 Synthesis

4.1.2.1 Synthesis of 2-(4-oxo-2-thioxothiazolidin-3-yl)-*N*-substituted acetamides

Method A

An equivalent of 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid **1** (1 mmol) together with 1-hydroxybenzotriazole (1.1 equivalents of HOBt hydrate) and 1 equivalent of corresponding aniline were dissolved in a dichloromethane/ethyl acetate 1:1 mixture (8 mL). After complete dissolution of this mixture, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC; 1.2 equivalents) was added in one portion. The mixture was stirred at room temperature for 8 hours. The reaction was monitored using TLC. Then, after the solution evaporated till dryness, ethyl acetate was added. Resulting suspension was washed twice with 0.1 M aq. HCl, 5% aq. sodium bicarbonate, followed by brine. The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated under the reduced pressure to initiate crystallization. *n*-Hexane was added to promote the precipitation. It was kept for 24 hours at +4 °C and the

precipitates were filtered off to give afforded amides **2**. If necessary, they were recrystallized from ethyl acetate.

Method B [25]

A solution of the acid **1** (1 mmol) and 4-nitroaniline (1 mmol) in pyridine (6 mL) was cooled at -10 °C, and phosphorus chloride (1.1 mmol) was added dropwise under vigorous stirring. The reaction mixture was stirred for 2 h, then the reaction was evaporated till dryness and DCM was added. Resulting mixture was washed successively twice with 0.1 M aq. HCl, 5% aq. sodium bicarbonate, followed by brine. The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated under reduced pressure and *n*-hexane was added to start crystallization. It was kept for 24 hours at +4 °C and the precipitate was filtered off to give 4-nitroanilide **2f**.

2-(4-Oxo-2-thioxothiazolidin-3-yl)-*N*-phenylacetamide [2] **2a** (method A). Brownish solid; yield 95%; mp 165-168 °C. IR (ATR): 3289 (NH), 1739 (C=O), 1661 (CO-NH), 1192 (C-N) cm^{-1}. Anal. Calcd. for $C_{11}H_{10}N_2O_2S_2$ (266.33): C, 49.61; H, 3.78; N, 10.52. Found: C, 46.80; H, 3.68; N, 10.49.

N-(4-Chlorophenyl)-2-(4-oxo-2-thioxothiazolidin-3-yl)acetamide **2b** (method A). Brownish solid; yield 91%; mp 177.5-180 °C. IR (ATR): 3299 (NH), 1739 (C=O), 1660 (CO-NH), 1192 (C-N) cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 10.48 (1H, s, NH), 7.55 (2H, d, *J* = 8.8 Hz, H2´, H6´), 7.37 (2H, d, *J* = 8.8 Hz, H3′, H5′), 4.70 (2H, s, NC$\underline{H_2}$CONH), 4.41 (2H, s, SC$\underline{H_2}$CON). ^{13}C NMR (125 MHz, DMSO): δ 203.26, 174.11, 163.61, 137.56, 128.95, 127.36, 120.86, 46.67, 36.24. Anal. Calcd. for $C_{11}H_9ClN_2O_2S_2$ (300.78): C, 43.92; H, 3.02; N, 9.31. Found: C, 43.78; H, 3.10; N, 9.12.

2-(4-Oxo-2-thioxothiazolidin-3-yl)-*N*-[4-(trifluoromethyl)phenyl]acetamide **2c** (method A). Yellowish solid; yield 76%; mp 107-109.5 °C. IR (ATR): 3276 (NH), 1741 (C=O), 1665 (CO-NH), 1186 (C-N) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.82 (1H, bs, NH), 7.63 (2H, d, *J* = 8.4 Hz, H3´, H5´), 7.54 (2H, d, *J* = 8.4 Hz, H2′, H6′), 4.84 (2H, s, NC$\underline{H_2}$CONH), 4.10 (2H, s, SC$\underline{H_2}$CON). ^{13}C NMR (126 MHz, $CDCl_3$): δ 201.02, 173.35, 163.44, 140.49 (q, *J* = 1.9 Hz), 127.65 (q, *J* = 32.3 Hz), 126.21 (q, *J* = 3.6 Hz), 123.97 (d, *J* = 272.5 Hz), 119.59, 46.98, 35.73. Anal. Calcd. for $C_{12}H_9F_3N_2O_2S_2$ (334.34): C, 43.11; H, 2.71; N, 8.38. Found: C, 43.12; H, 3.00; N, 8.41.

2-(4-Oxo-2-thioxothiazolidin-3-yl)-*N*-[4-methylphenyl]acetamide **2d** (method A). Yellow solid; yield 85%; mp 170.5-172.0 °C. IR (ATR): 3290 (NH), 1740 (C=O), 1654 (CO-NH), 1187 (C-N) cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 10.23 (1H, s, NH), 7.40 (2H, d, *J* = 8.4 Hz, H2´, H6´), 7.11 (2H, d, *J* = 8.4 Hz, H3′, H5′), 4.68 (2H, s, NC$\underline{H_2}$CONH), 4.40 (2H, s, SC$\underline{H_2}$CON), 2.23 (3H, s, CH_3). ^{13}C NMR (125 MHz, DMSO): δ 203.28, 174.13, 163.12, 136.21, 132.72, 129.38, 119.30, 46.61, 36.20, 20.60. Anal. Calcd. for $C_{12}H_{12}N_2O_2S_2$ (280.37): C, 51.41; H, 4.31; N, 9.99. Found: C, 51.31; H, 4.34; N, 10.01.

N-(4-Methoxyphenyl)-2-(4-oxo-2-thioxothiazolidin-3-yl)acetamide **2e** (method A). Brownish solid; yield 89%; mp 177-179.5 °C. IR (ATR): 3259 (NH), 1733 (C=O), 1659 (CO-NH), 1191 (C-N) cm^{-1}. ^{1}H NMR (500 MHz, DMSO): δ 10.17 (1H, s, NH), 7.42 (2H, d, *J* = 9.0 Hz, H2´, H6´), 6.97 (2H, d, *J* = 9.0 Hz, H3′, H5′), 4.66 (2H, s, NC$\underline{H_2}$CONH), 4.39 (2H, s, SC$\underline{H_2}$CON), 3.71 (3H, s, OCH_3). ^{13}C NMR (125 MHz, DMSO): δ 203.57, 174.44, 163.17, 155.89, 132.03, 121.14, 114.41, 55.62,

46.84, 36.50. Anal. Calcd. for $C_{12}H_{12}N_2O_3S_2$ (296.37): C, 48.63; H, 4.08; N, 9.45. Found: C, 48.77; H, 4.00; N, 9.59.

N-(4-Nitrophenyl)-2-(4-oxo-2-thioxothiazolidin-3-yl)acetamide **2f** (method B). Yellow solid; yield 78%; mp 173.5-176 °C. IR (ATR): 3300 (NH), 1743 (C=O), 1685 (CO-NH), 1189 (C-N) cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 8.78 (1H, s, NH), 8.20 (2H, d, *J* = 9.1 Hz, H3´, H5´), 7.70 (2H, d, *J* = 9.1 Hz, H2′, H6′), 4.87 (2H, s, NC$\underline{H_2}$CONH), 4.14 (2H, s, SC$\underline{H_2}$CON). ^{13}C NMR (75 MHz, DMSO): δ 200.89, 173.23, 163.21, 144.01, 142.75, 125.13, 119.42, 47.18, 35.75. Anal. Calcd. for $C_{11}H_9N_3O_4S_2$ (311.34): C, 42.44; H, 2.91; N, 13.50. Found: C, 42.32; H, 3.09; N, 13.41.

4.1.2.2 Synthesis of 2-(4-oxo-2-thioxothiazolidin-3-yl)acetates

An equivalent of 2-(4-oxo-2-thioxothiazolidin-3-yl)acetic acid **1** (1 mmol) together with 1 equivalent of corresponding phenol, 1 equivalent of triethylamine and 4-(dimethylamino)pyridine (0.1 eq.) were dissolved in DCM (7 mL). After complete dissolution, EDC (1.2 equivalents) was added in one portion. The mixture was stirred at room temperature for 8 hours. The reaction was monitored using TLC. The reaction mixture was washed three times with 0.1 M aq. HCl, 10% aq. sodium carbonate, followed by brine. The organic layer was dried over sodium sulfate and filtered. Active charcoal was added to the boiling solution to decolorize it. After filtration and cooling down of the mixture, crystallization was initiated by the addition of *n*-hexane. It was kept for 24 hours at +4 °C and the precipitates were filtered off to give esters **3**. If necessary, they were recrystallized from ethyl acetate.

Phenyl 2-(4-oxo-2-thioxothiazolidin-3-yl)acetate **3a**. Brownish solid; yield 70%; mp 110.5-113 °C. IR (ATR): 1772 (OCO), 1731 (C=O) cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.41-7.37 (2H, m, H3´, H5´), 7.26 (1H, dt, *J* = 7.5 Hz, *J* = 1.2 Hz, H4′), 7.14-7.10 (2H, m, H2′, H6′), 4.97 (2H, s, NC$\underline{H_2}$COO), 4.11 (2H, s, SC$\underline{H_2}$CON). ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.33, 172.94, 164.48, 150.16, 129.52, 126.35, 121.22, 44.91, 35.71. Anal. Calcd. for $C_{11}H_9NO_3S_2$ (267.32): C, 49.42; H, 3.39; N, 5.24. Found: C, 49.60; H, 3.51; N, 5.33.

4-Chlorophenyl 2-(4-oxo-2-thioxothiazolidin-3-yl)acetate **3b**. Brownish solid; yield 73%; mp 133-135 °C. IR (ATR): 1774 (OCO), 1745 (C=O) cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.32-7.36 (2H, m, H2´, H6´), 7.08-7.03 (2H, m, H3′, H5′), 4.95 (2H, s, NC$\underline{H_2}$COO), 4.11 (2H, s, SC$\underline{H_2}$CON). ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.29, 172.89, 164.32, 148.57, 131.83, 129.60, 122.62, 44.83, 35.73. Anal. Calcd. for $C_{11}H_8ClNO_3S_2$ (301.77): C, 43.78; H, 2.67; N, 4.64. Found: C, 43.67; H, 2.58; N, 4.62.

4-(Trifluoromethyl)phenyl 2-(4-oxo-2-thioxothiazolidin-3-yl)acetate **3c**. Brownish solid; yield 66%; mp 101-102.5 °C. IR (ATR): 1776 (OCO), 1748 (C=O) cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 7.67 (2H, d, *J* = 8.5 Hz, H3′, H5′), 7.26 (2H, d, *J* = 8.5 Hz, H2´, H6´), 4.99 (2H, s, NC$\underline{H_2}$COO), 4.14 (2H, s, SC$\underline{H_2}$CON). ^{13}C NMR (125 MHz, DMSO): δ 200.26, 172.86, 164.07, 152.49 (q, *J* = 1.4 Hz), 128.70 (q, *J* = 32.9 Hz), 126.91 (q, *J* = 3.8 Hz), 123.66 (d, *J* = 272.7 Hz), 121.80, 44.81, 35.74. Anal. Calcd. for $C_{12}H_8F_3NO_3S_2$ (335.32): C, 42.98; H, 2.40; N, 4.18. Found: C, 43.14; H, 2.57; N, 3.94.

4-Methylphenyl 2-(4-oxo-2-thioxothiazolidin-3-yl)acetate **3d**. Brownish solid; yield 78%; mp 140-142.5 °C. IR (ATR): 1751 (OCO), 1741 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.20-7.13 (2H, m, H2´, H6´), 7.02-6.95 (2H, m, H3′, H5′), 4.94 (2H, s, $NC\underline{H_2}COO$), 4.10 (2H, s, $SC\underline{H_2}CON$), 2.34 (3H, s, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 200.34, 172.94, 164.62, 147.91, 136.05, 129.98, 120.85, 44.90, 35.70, 20.83. Anal. Calcd. for $C_{12}H_{11}NO_3S_2$ (281.35): C, 51.23; H, 3.94; N, 4.89. Found: C, 51.03; H, 3.86; N, 4.96.

4-Methoxyphenyl 2-(4-oxo-2-thioxothiazolidin-3-yl)acetate **3e**. Brownish solid; yield 76%; mp 111-112.5 °C. IR (ATR): 1753 (OCO), 1744 (C=O) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.04-7.00 (2H, m, H3´, H5´), 6.90-6.85 (2H, m, H2′, H6′), 4.93 (2H, s, $NC\underline{H_2}COO$), 4.10 (2H, s, $SC\underline{H_2}CON$), 3.78 (3H, s, OCH_3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.34, 172.95, 164.82, 157.59, 143.64, 122.01, 114.49, 55.59, 44.90, 35.71. Anal. Calcd. for $C_{12}H_{11}NO_4S_2$ (297.35): C, 48.47; H, 3.73; N, 4.71. Found: C, 48.59; H, 3.78; N, 4.90.

4-Nitrophenyl 2-(4-oxo-2-thioxothiazolidin-3-yl)acetate **3f**. Brownish solid; yield 79%; mp 145-146.5 °C. IR (ATR): 1778 (OCO), 1737 (C=O) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.30-8.25 (2H, m, H3´, H5´), 7.33-7.29 (2H, m, H2′, H6′), 4.99 (2H, s, $NC\underline{H_2}COO$), 4.14 (2H, s, $SC\underline{H_2}CON$). ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.24, 172.83, 163.79, 154.59, 145.76, 125.32, 122.24, 44.77, 35.78. Anal. Calcd. for $C_{11}H_8N_2O_5S_2$ (312.32): C, 42.30; H, 2.58; N, 8.97. Found: C, 42.15; H, 2.42; N, 8.83.

4.2 Determination of cholinesterases inhibition

The IC_{50} values were determined using the spectrophotometric Ellman's method, which is a simple, rapid and direct method to determine the SH and -S-S- group content in proteins [26]. This method is widely used for the evaluation of cholinesterase activity and screening the efficiency of ChE inhibitors. Cholinesterase activity is measured indirectly by quantifying the concentration of the 5-thio-2-nitrobenzoic acid ion formed in the reaction between the thiol reagent 5,5'-dithiobis(2-nitrobenzoic acid) and thiocholine, a product of substrate hydrolysis (*i.e.*, acetylthiocholine) by cholinesterases [34]. All of the tested compounds were dissolved in 0.01 M dimethyl sulphoxide and then diluted in demineralised water to 0.001 M. Ellman's method was modified slightly according to Zdrazilova et al. [35].

Acetylcholinesterase was obtained from electric eel (*Electrophorus electricus* L.) and butyrylcholinesterase was from equine serum. Galantamine and rivastigmine were involved as reference drugs. All of the enzymes, galantamine and rivastigmine were purchased from Sigma-Aldrich (St. Louis, USA).

4.3 Determination of octan-1-ol/water partition coefficients, drug-likeness and polar surface area

The values of partition coefficient as quantitative descriptors of lipophilicity were determined using a traditional shake-flask method [36,37,38]. First, the two solvents were mutually saturated at the temperature of the experiment (20 °C) in a way described in literature [38]. Then the determination of $\log P_{\text{octanol-water}}$ ($\log P_{ow}$) was performed subsequently: octan-1-ol (1.5 mL) and octan-1-ol solution of tested compound (10 µl, 0.01 M) were placed into the test tube. This mixture was intensively shaken for 15 min. Then 1 mL of this mixture was placed into the quartz cuvette and its absorbance at the absorption maximum wavelength (255 nm) was measured. Thereby the value of absorbance

corresponding to 100 % of the tested compound in octanol was obtained. The reference solution was octanol. Into the other test tube octanol (1.5 mL), water (1.5 mL) and octanol solution of tested compound (10 µl, 0.01 M) were placed. This mixture was intensively shaken for 15 min and then centrifuged (3000 rpm, 10 min). Then 1 mL of octanol layer was placed into the quartz cuvette and its absorbance at the absorption maximum wavelength (255 nm) was measured. The reference solution was octanol again. Thereby the percentage content of tested compound in the octanol layer (%) was obtained. Subsequently the logP_{ow} (logP_{ow} = log(c_1/c_2), where c_1 and c_2 are molar concentrations of tested compound in octanol and water) was calculated. For each compound, at least two measurements were performed.

The calculated log*P* values, molar refractivity, molecular weights and polar surface areas were determined using the program CS ChemOffice Ultra version 15.0 (CambridgeSoft, Cambridge, MA, USA).

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Conflicts of Interest

The authors declare no conflict of interest.

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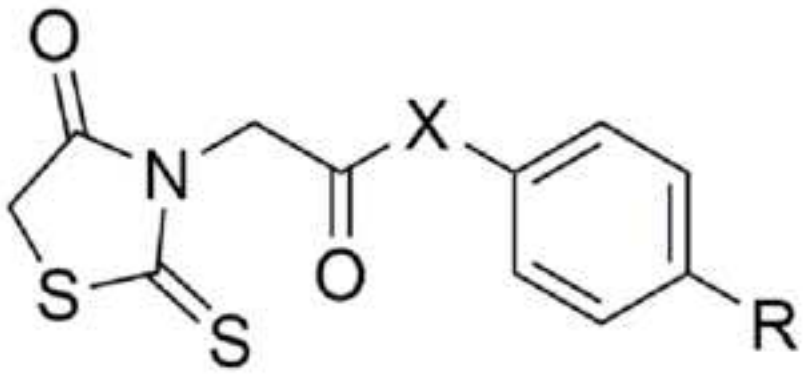

X = O, NH

R = H, Cl, CF_3, CH_3, OCH_3, NO_2

IC_{50} values for acetylcholinesterase from 24.05 μmol/L

IC_{50} values for butyrylcholinesterase from 7.92 μmol/L

Highlights

- Esters and amides of rhodanine-3-acetic acid inhibit cholinesterases.
- IC_{50} values from 7.92 μM.
- Rhodanines produce balanced inhibition of butyrylcholinesterase and acetylcholinesterase.
- The most active inhibitors were derivatives of 4-nitroaniline/phenol.