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Repurposing *N,N'*-bis-(arylamidino)-1,4-piperazinedicarboxamidines: an unexpected class of potent inhibitors of cholinesterases

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Graphical Abstract:

Repurposing *N,N'*-bis-(arylamidino)-1,4-piperazinedicarboxamidines: an unexpected class of potent inhibitors of cholinesterases

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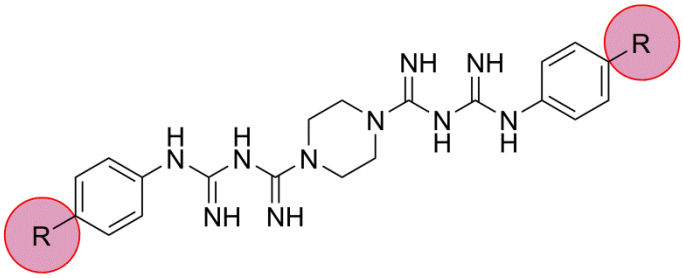


R = NO_2: K_i = 0.52 μM (AChE), K_i = 10.78 μM (BChE)
R = ^{t}Bu: K_i = 1.41 μM (AChE), K_i = 0.12 μM (BChE)
Galantamine.HBr: K_i = 2.07 μM (AChE), K_i = 0.54 μM (BChE)

Abstract

Drug repurposing (= drug repositioning) is an effective way to cut costs for the development of new therapeutics and to reduce the time-to-market time-span. Following this concept a small library of compounds was screened for their ability to act as inhibitors of acetyl- and butyrylcholinesterase. Picloxydine, an established antiseptic, was shown to be an inhibitor for both enzymes. Systematic variation of the aryl substituents led to analogs possessing almost the same good properties as gold standard galantamine hydrobromide.

Keywords: picloxydine; acetylcholinesterase; butyrylcholinesterase; bisbiguanides

1. Introduction

The development of a new drug is costly, and on average it takes about US$ 2-3 billion or more to develop a new therapeutic. This process is also time-consuming, and the time-span from target discovery to approval is about 12-16 years. The development of a new therapeutic is complex since the failure rate during this process is about 95%.[1]

Several years ago, the concept of “repurposing” (also known as “repositioning”) has been invented. This concept extends the “off-label-prescription” [2] already being in use for many years. The concept of repurposing improves the development of new treatments by creating new uses for “older” drugs that are already in use for another disease or have passed several key-steps along the development paths.[1, 3] A successful example for drug repurposing comprises the use of thalidomide for treatment of leprosy [4] or several malignant tumors [5, 6]. Last but not least it is worth to mention the more or less serendipitous discovery of sildenafil for treating men suffering from erectile dysfunction.[7, 8]

The number of people with age-related diseases such as Alzheimer’s disease (AD) grows continuously. AD causes progressive loss of memory, and worldwide nearly 44 million to 46.8 million [9,10] persons are affected. Applying a repurposing concept early in 2015 (from a cooperation of the US based National Center for Advancing Translational Science, NCATS, and Yale University School of Medicine) antitumor active saracatinib was found and successfully tested in mice for treating AD.[11] Presently AD cannot be cured but the quality of life for AD patients (or patients suffering from Myasthenia gravis pseudoparalytica) is

significantly improved upon treatment with inhibitors of cholinesterases (ChE) such as donezepil (reversible ChE inhibitor), galantamine (reversible ChE inhibitor), or rivastigmine (irreversible ChE inhibitor).[12-16]

2. Results and discussion

2.1. Synthesis

Following this concept we screened a small library of established drugs for their ability to inhibit enzymes of therapeutic interest, among them acetyl- and butyrylcholinesterase (AChE, BChE).

To our surprise picloxydine (**PD**, Fig. 1), an antiseptic, was shown to be a good inhibitor for BChE. **PD** is a bisbiguanide, and these compounds are known as useful microbiocides, insecticides or disinfectants.[17] Except for a very early report [18] about the inhibitory effect of paludrine (**PA**, a prophylactic antimalarial drug usually taken together with another antimalarial drug, Fig. 1) on cholinesterases, to our knowledge, the cholinesterase inhibiting effect of these compounds has not been investigated so far. Recently, cyclic acyl guanidines bearing extra carbamate moieties [19] and aryl-urea bnzofuranylthiazole hybrids [20] have been found to inhibit cholinesterases, too

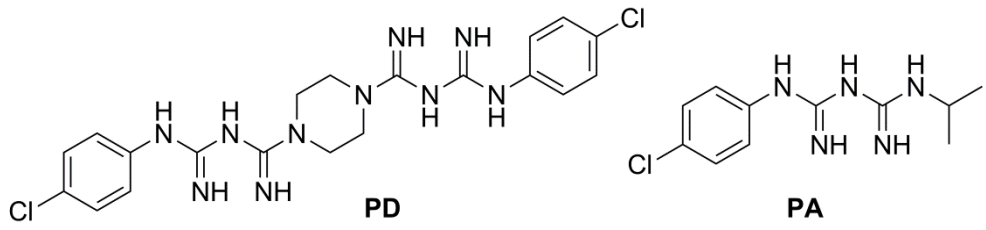
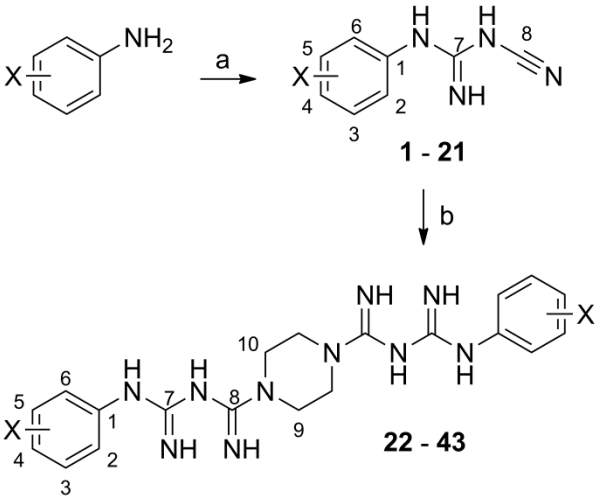


Fig. 1. Structure of antiseptic picloxydine (**PD**) and anti-malarial paludrine (**PA**)

As a consequence we decided to investigate bisbiguanides more detail. The synthetic route to bisbiguanides is depicted in Scheme 1.

Thus, commercially available (substituted) anilines were allowed to react at 60 °C with sodium dicyanamide in the presence of aq. hydrochloric acid for three hours,[21] and the corresponding *N*-aryl-*N'*-cyanoguanides **1-21** were obtained in good to excellent yields. From the microwaves assisted reaction (120 °C, 2 hours) of **1-21** with piperazine the

bis(arylamidino)-1,4-piperazine-dicarboxamids **22-43** were obtained. As exemplified for **22**, these compounds are characterized in their ^{13}C NMR spectra by the presence of two signals at δ = 159.7 ppm (assigned to the NHC(=NH)-piperazine fragment) and δ = 157.6 ppm [NH-C(=NH)] while the 4 carbons of the piperazine moiety were detected at δ = 44.9 ppm. In their ESI-MS spectra always a quasimolecular ion $[M+2H]^{2+}$ was detected.



Scheme 1. Synthesis of the target compounds **22-43** starting from substituted amines and dicyanamide: a) 1 eq. HCl (aq.), then 1 eq. $NaN(CN)_2$, 60 °C, 3 hours; b) 2 eq. HCl (aq.), piperazine (1 eq.), MeOH, then 4 eq. **1-20**, 120 °C, 2 h (microwave-assisted).

2.2. Biological screening and structure activity relationships

Compounds **22-43** were subjected to Ellman's test [22] to determine their ability to inhibit the enzymes AChE and BCh; the inhibition constants K_i (for competitive inhibition) and K_i' (for non-competitive inhibition) as well as the type of inhibition was determined by using Lineweaver-Burk plots, Dixon plots and Cornish-Bowden plots. The results from these assays are compiled in Table 1.

Table 1. Inhibitory constants for galantamine hydrobromide (**GH**) and compounds **22-43** (K_i (competitive inhibition) and K_i**'** (non-competitive inhibition) in μM), determined using Ellman`s assay employing acetylcholinesterase with galantamine hydrobromide (**GH**) as standard.

Compound	X	K_i (μM)	K_i' (μM)	Type of inhibition

GH		0.54±0.01		competitive
22	H	13.53±0.14	69.16±2.56	Mixed type
23	2-F	14.92±0.25	58.58±1.97	Mixed type
24	3-F	14.86±0.32	104.27±9.61	Mixed type
25	4-F	7.77±0.24	16.19±0.17	Mixed type
26	2-Cl	6.11±0.03	66.68±11.5	Mixed type
27	3-Cl	4.45±0.29	> 30	Mixed type
28	4-Cl	22.00±0.09	28.31±0.24	Mixed type
29	2,4'-Cl	9.34±0.79	75.30±5.69	Mixed type
30	2-Br	4.19±1.74	40.53±22.01	Mixed type
31	3-Br	6.17±0.51	25.40±2.22	Mixed type
32	4-Br	3.43±0.92	35.55±1.63	Mixed type
33	2-I	1.56±0.25	> 100	Mixed type
34	3-I	2.79±0.26	19.94±2.43	Mixed type
35	4-I	5.08±0.87	49.10±5.62	Mixed type
36	2-OMe	4.34±0.47	13.68±0.82	Mixed type
37	3-OMe	10.41±0.66	45.23±2.71	Mixed type
38	4-OMe	25.55±1.37	88.86±2.79	Mixed type
39	3,4,5-OMe	7.74±0.98	41.21±0.56	Mixed type
40	4-OCF_3	19.83±1.83	62.24±8.95	Mixed type
41	4-Me	9.25±0.04	> 100	Mixed type
42	4-*t*Butyl	1.41±0.06	21.77±1.06	Mixed type
43	4-NO_2	0.52±0.06	2.96±0.40	Mixed type

Table 2. Inhibitory constants for galantamine hydrobromide (**GH**) and compounds **22-43** (K_i (competitive inhibition) and K_i**'** (non-competitive inhibition) in μM), determined using Ellman`s assay employing butyrylcholinesterase with galantamine hydrobromide (**GH**) as standard.

Compound	**X**	**K_i (μM)**	**K_i' (μM)**	**Type of inhibition**
GH		2.07±0.29		competitive
22	H	0.95±0.04	> 40	Mixed type
23	2-F	6.82±1.32	25.49±10.06	Mixed type
24	3-F	1.70±0.01	32.85±2.80	Mixed type
25	4-F	1.18±0.16	15.18±1.59	Mixed type
26	2-Cl	1.85±0.34	12.70±3.82	Mixed type
27	3-Cl	0.74±0.08	12.24±4.04	Mixed type
28	4-Cl	0.62±0.03	9.37±0.09	Mixed type
29	2,4'-Cl	0.41±0.15	7.51±3.08	Mixed type

30	2-Br	1.25±0.05	12.34±3.83	Mixed type
31	3-Br	0.23±0.11	10.32±3.02	Mixed type
32	4-Br	0.61±0.09	3.84±0.32	Mixed type
33	2-I	1.96±0.09	11.19±0.13	Mixed type
34	3-I	0.21±0.02	3.04±1.37	Mixed type
35	4-I	0.31±0.12	2.74±0.69	Mixed type
36	2-OMe	5.20±0.17	33.96±0.93	Mixed type
37	3-OMe	3.02±0.01	54.84±3.43	Mixed type
38	4-OMe	1.78±0.13	35.13±4.97	Mixed type
39	3,4,5-OMe	9.03±0.51	---	competitive
40	4-OCF_3	3.34±0.23	> 20	Mixed type
41	4-Me	0.45±0.05	11.80±4.97	Mixed type
42	4-*t*Butyl	0.12±0.02	4.41±1.42	Mixed type
43	4-NO_2	10.78±0.81	22.87±4.79	Mixed type

To get a deeper insight into the mode of action of these compounds, some molecular modelling calculations were performed. The ligands contain protonable nitrogen atoms at three different moieties in the compounds. To decide which one are most likely protonated when binding to the enzymes, the proton affinities (for a single protonation only) were calculated. The results clearly predict that protonation at the piperazine ring is disfavored by 26.8 kcal/mol. Thus protonation at this site can be ruled out under physiological conditions in the active site of the enzymes. Despite a favored protonation at the second guanidinyl group by 9.7 kcal/mol (as compared to a protonation of the guanidinyl moiety close to the piperazine ring) both protonation options were taken into consideration during docking. Altogether three forms of each ligand were used for docking, the neutral unprotonated one and both symmetrically protonated at the guanidine moieties at both sides, *i.e*. double protonation.

The results from the calculations showed for all docking results, that the protonated forms of the ligands are clearly favored over the neutral form. This is due to the fact that in both enzymes there are two negatively charged amino acid residues (AchE: D74, E202; BChE:D70, E197) allowing the formation of salt bridges with the ligands. However, for AchE no docking arrangement could be observed where one of the protonated guanidino groups interacts with E202, whereas in BChE this appears quite often. There is an essential difference between the active sites of AChE and BChE. The gorge in AchE is much narrower than the one in BChE (Figure 2).

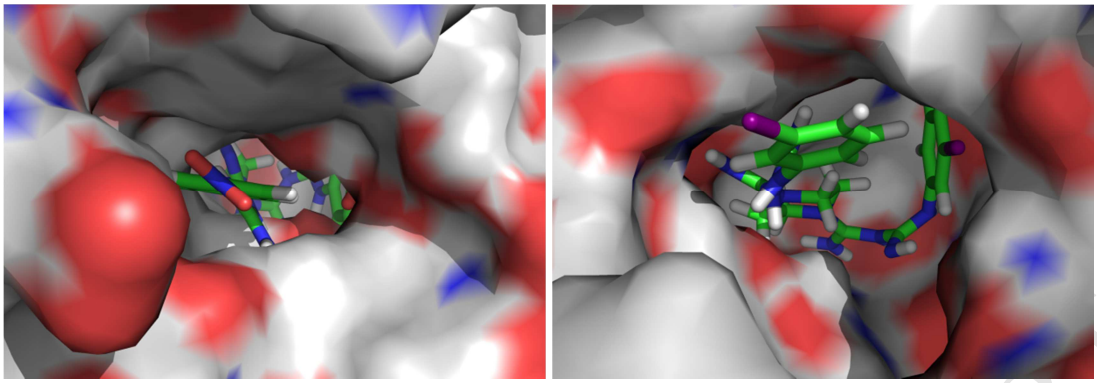


Fig. 2: Comparison of the active sites of AChE with bound **43** (*p*-NO_2, left) and BChE with bound **34** (*m*-I, right) from the top.

This striking difference is also reflected when comparing the most favored docking arrangements of the most active compounds displayed in Figure 3. Whereas for AChE in all poses the ligands adopt an almost extended conformation, for BChE the ligands are folded or bend because of the available space for optimal interactions. The most active compound for AchE inhibition is **43** which gave a significantly higher K_i value for BChE. In AChE one guanidino moiety forms a strong salt bridge with D74 and the other one a hydrogen bond with the backbone carbonyl of S293. Furthermore, a hydrogen bond is formed between the *p*-NO_2 group and the hydroxyl group of Y133. Molecular dynamics simulations over 5 ns in a water box applying periodic boundary condition proved the stability of the entire complex with only minor movements of the ligand in the active site. The presence of some water molecules (having been automatically placed in the active site) led to a further stabilization of the complex by bridging water hydrogen bonds between the guanidino groups and E202, E292 and N87.

All other compounds except the *p*-NO_2 derivative **43** were not able to form such a hydrogen bond with Y133; this obviously led to the reduction of their affinity to the enzyme. In BChE one guanidino group forms a salt bridge with E197, and W82 may form π–π interactions with the same moiety. In comparison to the binding pose with AchE there is only a (not perfect) salt bridge of one of the guanidino groups with E74 and a hydrogen bond with the carbonyl backbone of S293. Corresponding molecular dynamics simulations (performed as for 43 and AchE) resulted in only one new bridging water hydrogen bond with of one nitro-group with E197. There were no further stabilizing interactions as they have been discussed for the the *p*-NO2 substituted ligand.

One of the most active compounds for binding to BChE is **35** holding *p*-I substituents. The attractive interaction is favored by the formation of an almost perfect salt bridge between one guanidino group with E197 and hydrophobic interactions of the piperazine moiety with W82. Furthermore, one of the *p*-iodine substituents may form attractive interactions *via* the positive electrostatic potential of the sigma hole with the carbonyl group of side chain of N83 (red dashed line in Figure 3b). It is known, that the smaller the halogen atoms, the smaller is the sigma hole and thus the ability to form attractive interactions with lone pairs. This indeed correlates with the stepwise reduced affinity of the *p*-Br, *p*-Cl and *p*-F which has almost no positive sigma hole. The moderate activity of *p*-t-but substituted **42** will be discussed (vide infra) in comparison with it surprisingly high affinity to BChE. The docking arrangements in both AchE and BChE for the *meta* substituted iodine derivative **34** are very similar to the ones shown for the *p*-I substituted compound **35**. Thus, these calculations nicely explain the experimental findings.

Another very interesting compound is **42** holding two *tert*-butyl groups in the *para* positions. This compound is a good inhibitor of BChE but displays only weak inhibition for AChE. Calculations showed the formation of a salt bridge of one guanidino group with E197 but also the intra-molecular hydrophobic interaction between one of the *tert*-butyl moieties with the other aromatic ring system. This interaction highly supports the formation of a bend conformation resulting in binding almost perfectly into the active site. The preferred bend conformation for binding into the active site is also reflected by the much lower conformational force field energy (~ -80 kcal/mol) of the isolated bend conformation (like bound in BChE) in comparison to the extended one formed in AChE. For AChE the formation of a salt bridge of one guanidino group with D74 is observed and hydrophobic interactions of one *tert*-butyl group with the side chain of W86 support binding in the extended conformation.

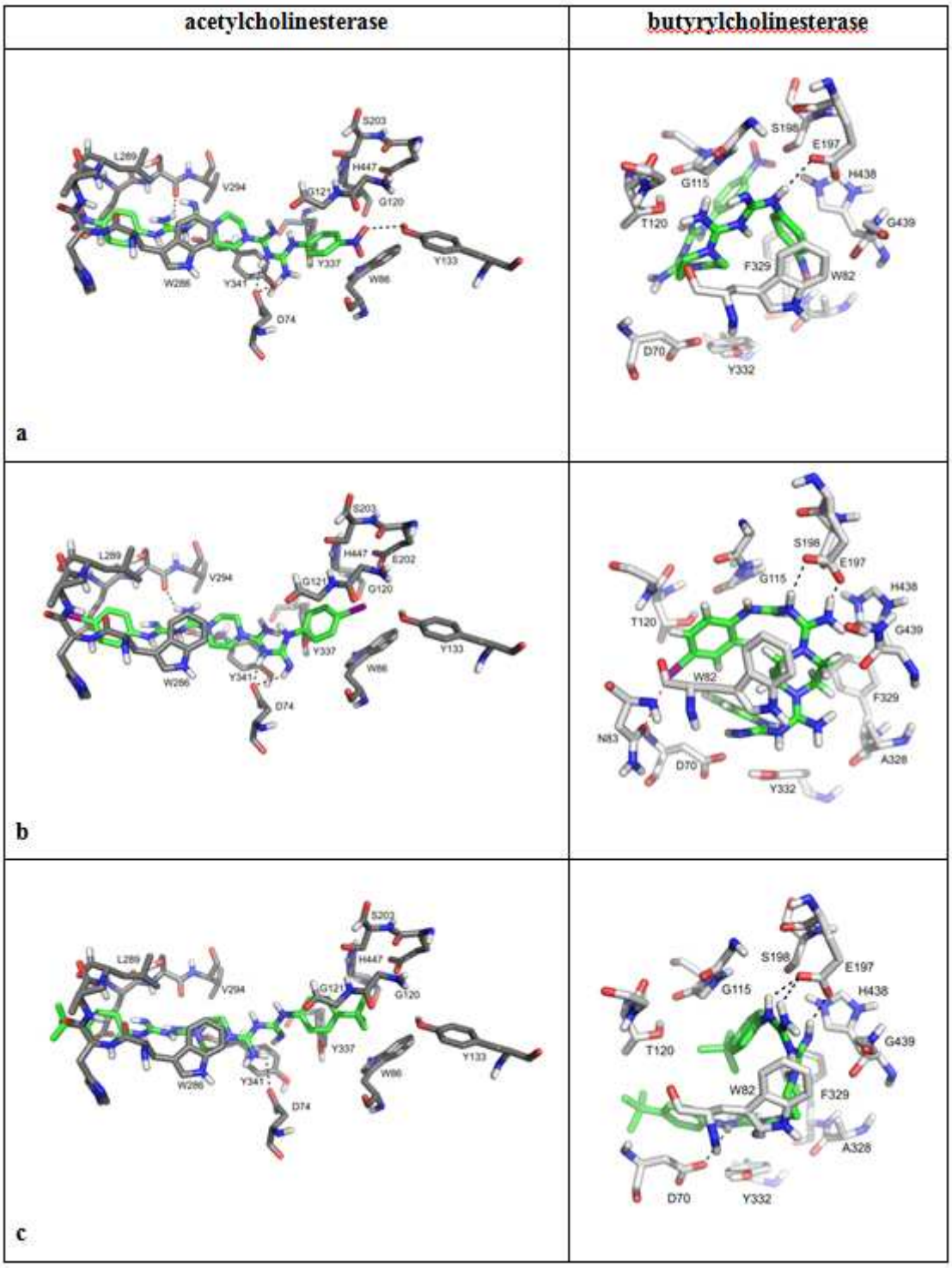


Fig. 3: Docking of the most favored arrangements of **a) 43** (*para*-NO_2), **b) 35** (*para*-I), and **c) 42** (*para*- *tert*-butyl), to the acetylcholinesterase (left) and to butyrylcholinesterase (right)

3. Conclusion

Following the concept of drug repurposing a small library of compounds was screened for their ability to act as inhibitor of enzymes being of interest in the treatment of diseases. Thus, picloxydine, an established antiseptic, was shown to be an inhibitor for acetyl- and butyrylcholinesterase. Systematic variation of the aryl substituents led to analogs possessing almost the same enzyme inhibiting properties as gold standard galantamine hydrobromide. While *p*-nitro substituted compound **43** was a good inhibitor for AChE, *p-tert*-butyl substituted bisbiguanide **42** inhibited BChE in the low μM range.

4. Experimental part

4.1.General

Detailed procedures for the synthesis, screening and modelling as well as a description of the equipment is given in the Supplementary material.

4.2. *General procedure for the synthesis of N-Cyano-N'-aryl- guanidines (**1-21**)*

To a solution of substituted aniline (22.49 mmol) in water (40 mL), hydrochloric acid (aq., 37 %, 1.9 mL, 22.49 mmol) and sodium dicyanamide (2.01 g, 22.49 mmol) were added. The mixture was stirred at 60 °C for three hours. After cooling to 0 °C for several hours, the crude product was filtered off and washed with ether.

4.3.*General procedure for the synthesis of N,N'-Bis-(arylamidino)-1,4-piperazinedicarboxamidines (**22-43**)*

To a solution of piperazine (100 mg, 1.16 mmol) in methanol (4 mL), hydrochloric acid (aq., 37%, 229 mg, 2.32 mmol) and the *N*-cyano-*N'*-aryl-guanidine **(1-21)** (4.64 mmol) were added. The reaction mixture was heated to 120 °C for two hours in a microwave reactor. After addition of methanol (4 mL) and aq. sodium hydroxide solution (1.16 mL, 2 M), the mixture was cooled to 0 °C, and the crude product **22-43** was collected by filtration and re-crystallized from methanol to yield pure **22-43** each as a colorless solid.

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Abbreviations

Acetylcholinesterase (AChE), butyrylcholinesterase (BChE), galantanine hydrobromide (GH), picloxydine (PD), paludrine (PA).

- Twenty one analogs of the antiseptic agent picloxydine were synthesized
- They were screened for their ability to inhibit cholinesterases AChE and BChE
- A *p*-nitro substituted bisbiguanide **43** was a good inhibitor for AChE
- A *p-tert*-butyl substituted analog **42**, however, inhibited BChE in the low μM range