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Design, synthesis, and characterization of novel, nonquaternary reactivators of GF-inhibited human acetylcholinesterase

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ABSTRACT

The goal of this research was to identify structurally novel, non-quaternarypyridinium reactivators of GF (cyclosarin)-inhibited hAChE that possess the capacity to mediate in vitro reactivation of GF-inhibited human acetylcholinesterase (hAChE). New compounds were designed, synthesized and assessed in GF-inhibited hAChE assays. Structure activity relationships for AChE binding and reactivation of GF-inhibited hAChE were developed. Lead compounds from two different chemical series, represented by compounds **17** and **38**, displayed proficient in vitro reactivation of GF-inhibited hAChE, while also possessing low inhibition of native enzyme.

Exposure to highly toxic organophosphorus compounds in the form of pesticides and their metabolites (e.g., paraoxon, malaoxon) or nerve agents (VX, Sarin, Soman or Cyclosarin) pose substantial threats to both military and civilian populations. Nerve agents of this type have been studied extensively and there is significant evidence showing their toxic effects are a result of phosphorylation of the active site serine residue in acetylcholinesterase (AChE).[1] Since AChE is responsible for hydrolyzing acetylcholine to choline, this irreversible inhibition of the AChE enzyme causes an accumulation of acetylcholine in both peripheral and central synapses, ultimately leading to deleterious effects, such as ocular pain, diminished vision, tightness of the chest, wheezing, bronchial constriction and secretion, and ultimately respiratory failure and death. Of significant relevance to our research efforts is the fact that nerve agents also penetrate the blood–brain barrier, inhibiting central AChE, thus causing a number of CNS related effects, such as confusion, ataxia, convulsions, slurred speech, and coma. Furthermore, studies have suggested that exposure to sub-lethal doses of nerve agent can dramatically impact CNS mediated behavioral attributes such as decreased ability to perform tasks.[2] Other studies have shown that the detrimental effects of a single dose of agent can be prolonged[3] and most of the human data, which is focused on organophosphate pesticides, suggest long-term CNS effects such as irritability, impaired memory, insomnia or anxiety.[4]

Pyridinium oxime compounds have been postulated to act as reactivators by binding to nerve agent-inhibited AChE and hydrolyzing the phosphonate group from the serine residue via nucleophilic attack of the oxime moiety on the phosphorus atom. The resulting reactivation of AChE allows the enzyme to continue converting acetylcholine to choline, helping maintain functional levels of acetylcholine.[5] Quaternary pyridinium aldoxime compounds, such as 2-PAM (Fig. 1) are limited to peripheral reactivation,[6] however, results with uncharged dihydropyrine 'pro-drugs' of 2-PAM have supported central protection.[7] Historically,[8] studies on non-pyridinium based AChE oxime reactivators have received little attention, with the exception of the simple acetyl oximes such as MINA and DAM.[9] During the course of our investigation reported here, a small number of non-pyridium based AChE reactivators were reported, represented by the dihydroisoquinoline **1**,[10] amidine **2**,[11] phenyl ketooxime **3**[12] and the amide oxime RS41A[13] (Fig. 1).

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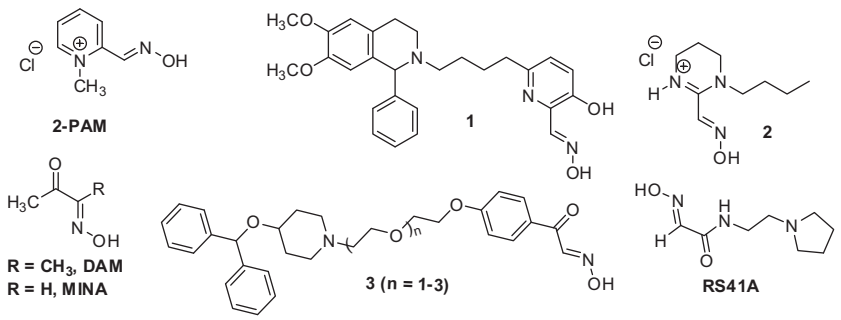


Figure 1. Structure of existing AchE reactivators.

Herein we report the design, synthesis and in vitro evaluation of a novel class of small molecule reactivators of GF-inhibited hAChE.[14] In all design iterations, several factors were taken into consideration. A balanced affinity for GF-inhibited AChE vs. uninhibited AChE is critical; however oxime pK_a has also been postulated to play a critical role for an efficient reactivation process.[15] Physicochemical property values (Log*D*, tPSA, MW, # hydrogen bond donors/acceptors) were determined and structures adjusted to maintain the desired range of values for CNS drug-like properties.[16] Based on these studies we chose to focus on two chemical series highlighted by the amide oxime analogs **9**–**22** (Fig. 2) and heteroaryl keto-oxime analogs **27**–**43** (Fig. 3). Both structural series allow for introduction of lipophilic amine templates, as well as various functional groups to modulate oxime properties.

The synthesis of the lipophilic amide oxime series is detailed below in Scheme 1. Treatment of primary amines with ethyl 2-(hydroxyimino)acetate provided the amide oxime analogs **4** in moderate yield (50–70%). The primary amines could also be treated with the 1,3-dioxin-4-one in toluene at 150 °C to produce the desired β-ketoamides **5**, which underwent subsequent oxime formation with sodium nitrite ($NaNO_2$), under cold, aqueous acidic conditions, which cleanly afforded the desired β-ketoamide oximes **6** in low (20–40%) yields. Finally, the cyanoamide analogs **8** were easily prepared in two steps via condensation with methyl-2-cyanoacetate, followed by oxime formation under *tert*-butyl nitrite/sodium propoxide conditions.

The synthesis route highlighted in Scheme 1 was used to prepare the analogs of various lipophilic amine templates as shown in Figure 2. The *N*-benzyl piperidine analogs **9**–**15** were prepared from the commercially available primary amines or the corresponding *N*-BOC protected piperidines. Analogs **16**–**18** and **19**–**22** were prepared from the corresponding dihydroisoquinoline[17] and aminotacrine[18] primary amines, respectively.

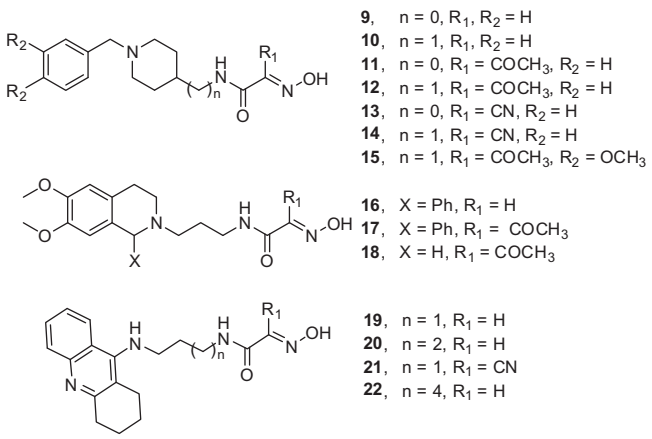


Figure 2. Structure of prepared amide oxime analogs

A representative synthetic route used to prepare the heteroaryl keto-oxime analogs is shown below in Scheme 2. Cyclic amino alcohol derivatives such as **23** were treated under reductive amination conditions with aldehydes and sodium triacetoxyborohydride to provide the tertiary amines **24** in good yields.[19] The acetyl pyridyl ethers **25** were prepared via palladium catalyzed coupling reaction of alcohol **24** and 2-bromo-acetyl pyridines in the presence of $Pd_2(dba)_3$/BINAP, which produced the desired pyridyl ketones in moderate yields.[20] Finally, oxime formation was accomplished under *t*-BuONO/KO*t*-Bu[21] conditions to provide the keto-oxime analogs **26** in low yields. Unfortunately, attempts to optimize the reaction conditions, as well as employ alternative conditions (i.e. TMSCl, NOCl)[22] were unsuccessful.

The synthetic route highlighted in Scheme 2 was used to prepare a number of analogs varying substitution on the piperidine and pyrrolidine rings, as well as the position of the keto-oxime on the pyridine or pyrimdine rings (Fig. 3).

In vitro screening for reactivation of GF-inhibited hAChE was assayed by a robotic spectrophotometric assay using acetylthiocholine as substrate at pH 7.4 and 25 °C.[23] The time-course of reactivation of GF-inhibited hAChE was determined by adding various concentrations of oxime to GF-inhibited hAChE. Samples of the oxime/inhibited AChE mixture were removed at sequential times to measure the oxime-induced recovery of AChE activity.

Table 1 summarizes two data points collected on each analog: (1) Corrected cumulative reactivation by 20 uM oxime and (2) Oxime/hAChE equilibrium constant for inhibition of hAChE. In our initial studies, we were gratified to see low levels of reactivation of GF-inhibited hAChE as a proof of concept for our new chemical matter in our piperidine amide oxime analogs **9** and **10**. Furthermore, the analogs also possessed weak inhibition of native enzyme. Incorporation of the β-ketoamide moiety (analog **12**) not only produced a significant increase in reactivation activity, but also maintained weak inhibition of native hAChE. A similar result was observed with the 3,4-dimethoxybenzyl analog **15**. Addition of the cyano group in compounds **13** and **14** resulted in a complete loss of reactivation activity. Replacement of the *N*-benzyl piperidine with the dihydroisoquinoline group delivered hAChE reactivators with very good reactivation levels and low inhibition of native enzyme as long as the β-ketoamide moiety was present (**17** and **18**). Finally, incorporation of the tacrine group as represented by compounds **19**–**22**, provided compounds with substantial inhibition of native enzyme, yet only low levels of reactivation, regardless of amide oxime substitution or length of the amine tether.

Structure activity relationship (SAR) studies on the heteroaryl keto-oxime series provided compounds with very good reactivation of GF-inhibited hAChE. Compounds **27**–**30** all possessed the *N*-benzyl-4-piperidine moiety and only differed by the substitution pattern of the ketooxime group on the pyridine ring, which had

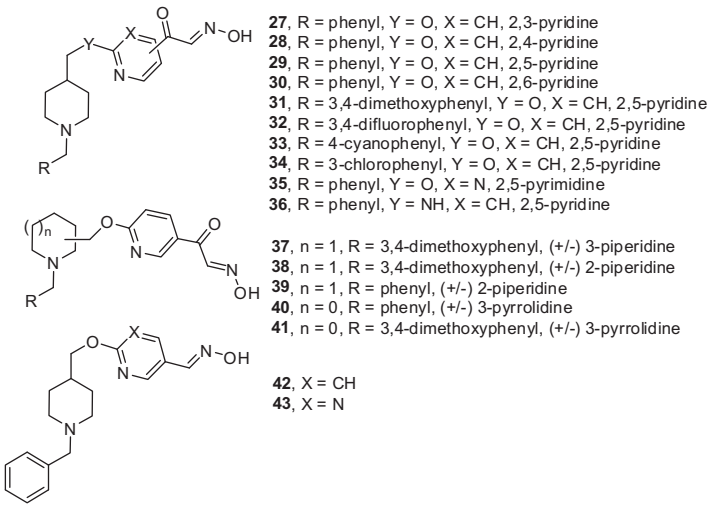
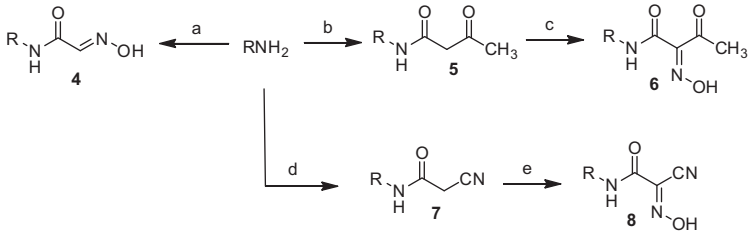
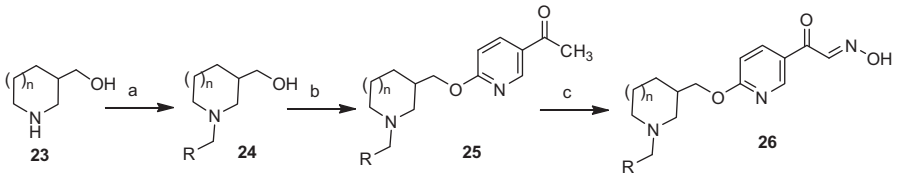


Figure 3. Structure of heteroaryl keto-oximes.



a) ethyl 2-(hydroxyimino)acetate, heat; b) 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one, toluene, 150 °C; c) $NaNO_2$, THF, $AcOH/H_2O$, 0°C to 20 °C; d) methyl 2-cyanoacetate, neat, 40 °C; e) t-butyl nitrite, NaOPr

Scheme 1. Synthesis of amide oxime analogs.



a) RCHO, $NaHB(OAc)_3$, DCM, >95%; 5-acetyl-2-bromopyridine, $Pd_2(dba)_3$, BINAP, Cs_2CO_3, 60%; c) t-BuONO, KOt-Bu, 30%.

Scheme 2. Synthesis of heteroaryl keto-oxime 26.

substantial effects on activity. The initial analog in this series, compound **29**, which possessed the 2,5-subsitution pattern on the pyridine, provided 23% reactivation and a K_i of 31 uM. Compound **30**, the 2,6-substituted pyridine, as compared to **29** showed a decrease in the affinity for native hAChE (199 μM vs 31 μM) as well as the corrected reactivation (7% vs 23%). Moving the keto-oxime group to the 2,4-position **28** decreased both reactivation (17%) and inhibition (144 uM) relative to **29**. However, the 2,3-substitution pattern in **27** displayed a marked improvement in reactivation (45%), while possessing low inhibition (180 uM). Based on these SAR findings the order of reactivation activity for substitution on the pyridine ring is 2,3- > 2,5- > 2,4- > 2,6. Changing the piperidine sidechain from benzyl **29** to 3,4-dimethoxybenzyl **31** produced equivalent reactivation activity, but showed a >60-fold decrease in affinity for native enzyme. Additional modifications to the *N*-benzyl side chain, as represented by compounds **32**–**34**, led to moderate improvements in reactivation activity with the 3,4-difluoro analog **32**. Installment of the 2,5-substituted pyrimidine ring **35**, produced compounds with very similar activity to the corresponding pyridine **29**. The ether linkage in **29** can also be replaced by an amine, resulting in a compound **36** with equivalent reactivation activity and a 9-fold decrease in affinity for native enzyme.

Finally, we attempted to translate SAR trends from the 4-substituted piperidines to the 2- and 3-substituted piperidines, as well as pyrrolidines, an effort which produced lead compounds with good activity. Compound **38** produced a significant increase in reactivation activity (65%) as compared to the corresponding 3-subsituted pipieridine **37** (38%) or the 4-substituted analog **29**. The 3-subsituted pyrrolidines **40** and **41** showed similar reactivation activity, however, as observed before, the 3,4-dimethoxybenzyl side chain displayed a decrease in affinity for native enzyme. Analogs **42** and **43**, which possess the oxime directly attached to the pyridine or pyrimidine ring, produced a significant decrease in reactivation relative to the corresponding keto-oximes **29** and **35**.[25]

Table 1
In vitro GF-Inhibited hAChE reactivation data[a]

Compound	Reactivation[b]	K_i[c]	Compound	Reactivation[b]	K_i[c]
MINA	7.7	0	**28**	17.2	144
9	7.5	2430	**29**	23.0	31.2
10	7.7	3178	**30**	7.3	199
11	11.8	234	**31**	25.3	1930
12	23.7	2300	**32**	30.8	159
13	0.1	732	**33**	21.4	1608
14	4.8	29938	**34**	18.9	298
15	22.7	1625	**35**	23.8	55.2
16	2.7	595	**36**	22.9	285
17	36.5	1926	**37**	38.2	345
18	28.9	2671	**38**	64.9	233
19	7.2	6.2	**39**	33.9	551
20	2.4	1.5	**40**	25.1	137
21	6.9	3.1	**41**	24.7	2517
22	0.8	0.59	**42**	0.4	270000
27	45.3	180	**43**	2.0	198

[a] Mean and STD data available in Supplementary information.
[b] % Corrected cumulative reactivation of GF-inhibited hAChE by 20 uM oxime at 4 h.[24]
[c] K_i inhibition constant for native AChE (uM).

In conclusion, two new chemical series of non-pyridinium based reactivators of hAChE have been discovered, both of which possess a range of reactivation activities for GF-inhibited hAChE and a varying degrees of selectivity over affinity for native enzyme. Lead compounds from these studies, such as **15**, **17**, **27**, **29** and **38**, which possess substantial reactivation activity, low affinity for native enzyme, and favorable physiochemical properties, have been advanced to blood brain barrier permeability, multiple in vivo reactivation, and agent-challenge studies. The results of these studies will be reported in due course.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.02.049.

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24. Reactivation at the 4 h time point was initially assessed due to slow reactivation rates observed with early analogs. Future work will involve testing the more advanced analogs at therapeutically relevant time points.
25. pKa's of various analogs were assessed via a capillary electrophoresis method (Analiza, Inc.) across a 24-point titration curve. pKa's for oximes **42** and **43** were higher (11.2 and 10.7, respectively) than the corresponding keto-oxime derivatives **29** and **35** (7.8 and 7.6, respectively).