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Antimicrobial, antimalarial and antileishmanial activities of mono- and bisquaternary pyridinium compounds

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Abstract

Pyridinium-based oxime compounds have been utilized worldwide as antidotes following exposure to anticholinesterase agents. In the event of combined chemical and biological incident, it is of vital importance to know the ability of antidotes to provide additional protection against biological threats. This paper reports results of *in vitro* antimicrobial and antiprotozoal activities of a series of quaternary pyridinium oximes against a number of lower pathogenicity BSL-1 and 2 agents. In general, our compound panel had little to no antimicrobial action except for thiophene- and benzothiophene-substituted monoquaternary pyridinium compounds **21** and **24** that showed moderate antibacterial activity against *Staphylococus aureus* and methicillin resistant *S. aureus* with IC_{50} values ranging from 12.2–17.7 μg/mL. Compounds **21** and **24** also exhibited antileishmanial activity against *Leishmania donovani* with IC_{50} values of 19 and 18 μg/mL, respectively. Another monoquaternary pyridinium compound with a bromobutyl side chain **17** showed antimalarial activity against both a chloroquine sensitive and resistant strains of *Plasmodium falciparum* with IC_{50} values of 3.7 and 4.0 μg/mL, respectively. None of the bisquaternary pyridinium compounds showed antimicrobial, or antiprotozoal activity. None of the compounds showed cytotoxic effects towards mammalian kidney fibroblasts. Results of this study indicate that the pyridinium compounds, some of which are already in use as antidotes, do not have significant antimicrobial and antiprotozoal activities and cannot be relied upon for additional protection in the event of combined chemical-biological incident.

Keywords

Pyridinium oximes; antimalarial; antimicrobial; antileishmanial; biological threats

Quaternary pyridinium bearing an oxime functional group are well known for their acetylcholinesterase reactivation activities and are used as antidotes in organophosphorus (OP) poisoning (1,2). Examples of well known AChE reactivators are pralidoxime, HI-6, trimedoxime and obidoxime. In the event of combined chemical-biological incident, it is of prime importance to provide antidotes against both chemical as well as biological threats. Quaternary pyridinium oximes are widely used as antidotes for chemical (nerve agent) poisoning but the ability of these antidotes to provide additional support against biological threats is unknown. Thus, it is important to know whether pyridinium oxime antidote provides additional protection against biological threats besides their AChE reactivation

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Supplementary material
Chemical structures of all mono- (**16–34**) and bis- (**35–61**) quaternary pyridinium oximes.

ability. Therefore, herein our aim was to screen series of quaternary pyridinium oximes with known AChE reactivation ability for their ability to provide protection against biological threats.

Besides this main objective, literature reports on interesting antimicrobial and antiprotozoal activities of several quaternary pyridinium class of compounds (3) prompted us to evaluate pyridinium class of AChE reactivators for these activities. Quaternary salts exhibit antimicrobial properties by adsorption to the cell wall of microorganisms, and bind to other biochemical components causing disruption of cellular processes which leads to cessation of bacterial growth and thus cell death (3). Chemical structures and IC_{50}/ MIC/ MBC values of representative mono- and bis-quaternary pyridinium compounds with antimicrobial 1–9, (4–11) antimalarial 10–14 (12–16) and antileishmanial 15 (17) activities are shown in Figure 1 and Table 1 respectively.

Biosafety level 1 and 2 agents have been widely used as model organisms and recently Utrup and Frey (18) used several model organisms as surrogates of biological threat agents. These include *Bacillus subtilis* for *B. anthracis* (anthrax), *E. coli* for *Variola major* (Smallpox), *Pseudomonas fluorescens* for *Burkholderia pseudomallei* (melioidosis) (18). The present paper reports results of an initial screen of a series of quaternary pyridinium oximes (19,20) against a number of lower pathogenicity BSL-1 and 2 model organisms. Use of model organisms in the first stage screening saves on the time and expense of BSL-3 studies, and allows for a determination of which compounds and BSL-3 organisms are worth further exploration.

Experimental section

General procedure for synthesis of quaternary pyridinium compounds

Hydroxyiminomethylpyridine (1 mmol) and the alkylating agent (1.5 mmol) in solvent (20 mL) were stirred at 60–80 °C for 1–24 h. The mixture was cooled to rt, the precipitate collected, washed with acetone (3 × 20 mL), dried under vacuum, and characterized by ^{1}H NMR, ^{13}C NMR, ESI-MS and IR (19). Spectral data for representative compounds is as follows: Bromothiophen-2-yl)-methyl-2-hydroxyiminomethylpyridinium chloride (**21**): Brownish black viscous oil; yield: 74%; ^{1}H NMR (400 MHz, CD_3OD): δ 8.82 (t, *J* = 7.2 Hz, 1H), 8.64 (m, 1H), 8.44 (t, *J* = 12 Hz, 1H), 8.27 (d, *J* = 8.0 Hz, 1H), 8.09 (m, 1H), 7.19 (d, *J* = 4.0 Hz, 1H), 7.06 (d, *J* = 4.0 Hz, 1H), 6.28 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD): δ 147.4, 146.2, 142.0, 141.4, 141.0, 131.0, 128.0, 127.5, 126.9, 125.7, 56.4; ESI-MS: *m/z* 296.95 $[M]^+$ (calcd for $[C_{11}H_{10}BrN_2OS]^+$ 297.97); 1-(Benzothiophene-3-yl)-methyl-2-hydroxyiminomethylpyridinium chloride (**24**): Greenish black viscous oil; yield: 64%; ^{1}H NMR (400 MHz, CD_3OD): δ 8.96 (s, 1H), 8.67 (s, 1H), 8.51 (m, 2H), 8.04 (m, 1H), 7.92 (m, 1H), 7.85 (m, 1H), 7.51 (s, 1H), 7.44 (m, 2H), 6.30 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD): δ 148.6, 146.1, 145.9, 145.7, 143.1, 141.8, 140.9, 127.9, 127.7, 126.4, 125.5, 125.1, 123.1, 121.2, 56.3; ESI-MS: *m/z* 269.1 $[M]^+$ calculated for $[C_{15}H_{13}N_2OS]^+$.

Biological testing

Antimicrobial, antimalarial, antileishmanial activities and cytotoxicity testing were performed using the protocols as reported by Samoylenko *et al* (21).

Results and Discussion

Mono- (**16–34**) and bisquaternary (**35–61**) pyridinium compounds used in this study were synthesized using a general synthetic strategy as depicted in Figure 2 and were characterized by ^{1}H NMR, ^{13}C NMR and ESIMS spectral data (19,22). All synthesized compounds were screened for *in vitro* antimicrobial, antimalarial, antileishmanial and cytotoxic activities.

Susceptibility testing for antifungal and antibacterial activity was done using a modified CLSI (formerly NCCLS) methods (21). Organisms include the fungi *Candida albicans*, *C. glabrata*, *C. krusei*, *Aspergillus fumigates*, *Cryptococcus neoformans* and the bacteria *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRS), *Pseudomonas aerugenosa*, *Escherichia coli*. Ciprofloxacin (0.07 and 0.08 μg/ml for *S. aureus* and MRS) and Amphotericin B (IC_{50} 0.76 μg/ml for *Cryptococcus neoformans*) were used as reference standards. Susceptibility of *Mycobacterium intracellulare* was done using the modified Alamar Blue procedure of Franzblau et al (23). Of all compounds tested, only monoquaternary pyridinium compounds **21** and **24** showed moderate antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* (MRS): compound **21**: IC_{50} 15.3 and 14.2 μg/mL; compound **24**: IC_{50} 12.2 and 17.7 μg/mL for *S. aureus* and MRS, respectively (Table 2). Both compounds **21** and **24** were inactive against all other test organisms. All other compounds showed no *in vitro* antifungal or antibacterial activity. Similar observations in the activity profile has been reported by Sidorchuk *et al* for quaternary quinolinium salts (7).

Although compounds **33**, **45**, **46**, **50–53**, **58**, **60** and **61** showed some antifungal activity against *Cryptococcus neoformans* with $IC_{50} > 20$ μg/mL, none of the compound showed significant antifungal activity against any of the organisms. *In vitro* antimalarial activity was evaluated against chloroquine sensitive (D6) and chloroquine resistant (W2) clones of *P. falciparum*. Determination of *in vitro* antimalarial activity was based on the determination of plasmodial LDH activity (24). Compounds were tested at three concentrations 4.76, 1.59 and 5.29 μg/mL. Chloroquine and artemisinin were used as reference standards. Monoquaternary pyridinium compound **17** showed antimalarial activities against both the clones of *Plasmodium falciparum* with the IC_{50} of 3.7 (D6 clone) and 4.0 (W2 clone) μg/mL with selectivity index of more than 1.3 (Table 3). None of the other compounds were active.

Antileishmanial activity against *L. donovani* promastigotes was determined by the Alamar Blue assay (25,26). Monoquaternary pyridinium compounds bearing thiophene functionality **21** and **24** showed antileishmanial activity against *L. donovani* with IC_{50} of 19 and 18 μg/mL, respectively (Table 3). None of the compounds were found to have any cytotoxic effects towards mammalian kidney fibroblasts (vero cells) (27).

Although a series of pyridinium compounds tested in the present study varied in structural features to allow for a critical study of structure-activity relationships, but this class of compound was almost devoid of any significant antimicrobial, antimalarial or antileishmanial properties. None of the compound demonstrated any activities comparable to standards used in the study. Mainly, bisquaternary pyridinium oximes displayed a complete lack of activity. Therefore, a detailed, meaningful analysis of structure-activity relationships was not possible. The only active compounds were two monoquaternary compounds **21** and **24** possessing a thiophene and banzothiophene linker chains with antibacterial and antileishmanial activities and another monoquaternary compound **17** with antimalarial activity. It is noteworthy to mention that compounds **21** and **24** which were already reported to possess AChE reactivation ability (19) also exhibited moderate antimicrobial and antileishmanial activities. In contrast, commercially used bisquaternary pyridinium antidotes trimedoxime (**42**) and obidoxime (**37**) with potent AChE reactivation abilities (19) were completely devoid of any antimicrobial or antiprotozoal activities. The major difference between reported antimicrobial / antimalarial quaternary pyridinium salts and compounds studied in this paper is that most of the compounds have long aliphatic chains (C_4–C_{20}) (Figure 1). This long aliphatic chain appears important for antimicrobial or antiprotozoal activity of quaternary salts. The poor or complete lack of activity of our compounds may be due to this difference. Thus, this work suggests that the aliphatic chain attached to pyridinium skeleton may be important for retaining antimicrobial/ antiprotozoal activity.

Merely, the presence of pyridinium skeleton does not suffice requirements to inhibit microbes or parasites.

Trimedoxime, obidoxime, HI-6 and several other oximes are known to be tolerated in high doses in humans and have been used as warfare agents for years (28,29). It was anticipated that the ability for safely achieving high serum concentrations of these compounds would allow for a significant antimicrobial effect against biological threats and might provide advantage in the event of a combined nerve agent/ biological-agent incident. Unfortunately, based on initial *in-vitro* studies in model organisms, the compounds appear to be tolerated as well by bacteria and protozoa as seen for mammals. Therefore, the utility of this class of compound does not extend to biological agents.

In conclusion, pyridinium oxime compounds which are widely used as antidotes for nerve gas poisoning, do not have ability to provide protection against microbial and protozoal infections. Results from this work provided important information about antidotes of OP poisoning that they cannot be relied upon for additional protection against biological threats in the event of combined chemical-biological incident.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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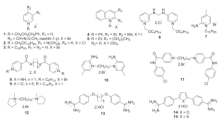


Figure 1.
Structures of antimicrobial (**1–6, 9**), antimalarial (**7, 8, 10–14**) and antileishmanial (**15**) pyridinium salts / dications

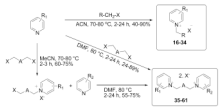


Figure 2.
General Scheme for synthesis of mono- (**16–34**) and bis- (**35–61**) quaternary pyridinium compounds (R_1 = CHNOH, $CONH_2$; R_2 = CHNOH; Position of R_1 or R_2: 2, 3 or 4; X = Br, Cl, OMs; R = CH_2CH_2Br, $CH_2CH_2CH_2Br$, heterocycles – thiophene, furan or isoxazole; A = O, CH_2, CH_2CH_2, heterocycles - thiophene-furan or isoxazole).

Table 1

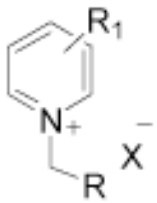
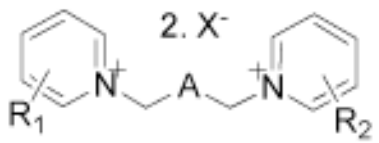
Antimicrobial and antiprotozoal activities of reported pyridinium quaternary compounds

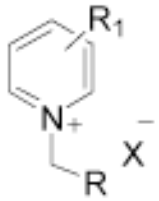
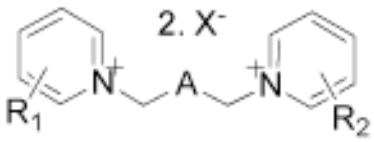
Entry	IC_{50}/ EC_{50}/ MIC/ MBC	Reference
1	MIC 9.8 (*S. aureus*), 39.1 (*E. coli*), 19.5 (*C. albicans*), and 9.8 (*E. faecalis*) μg/mL	(4)
2	MIC 1.4 (*S. aureus*), 11 (*C. albicans*), 6 (*B. subtilis*) and 22 (*E. coli*) μM	(5)
3	MBC 15.6 (*S. aureus*), 10.4 (*E. coli*), 16.3 (*E. faecalis*) μM	(6)
4 – 5	MBC 62.5 μg/mL (*S. aureus*, *S. albus* and MRS)	(7)
6	MIC 7 (*S. aureus*), 27 (*E. coli*), 13.5 (*C. albicans*) and 14 (*B. subtilis*) μM	(8)
7	IC_{50} 1.5 μM (*P. falciparum*)	(9)
8	EC_{50} 52 nM (FCR-3 - Chloroquine sensitive strain of *P. falciparum*)	(10)
9	MIC 1.1 (*S. aureus*), 1.4 (*MRS*), 1.4 (*B. subtilis*), 5.2 (*E. coli*) and 13.2 (*P. aeruginosa*) μM	(11)
10	IC_{50} 0.5 nM (*P. falciparum*)	(12)
11	EC_{50} 8 nM (*P. falciparum*)	(13)
12	IC_{50} 0.65 nM (*P. falciparum*)	(14)
13	IC_{50} 19 nM (*P. falciparum*)	(15,30)
14	IC_{50} 5.5 nM (*P. falciparum*)	(16,30)
15	IC_{50} 0.82 μM (*L. donovani*)	(17)

S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; C. albicans: Candida albicans; E. faecalis: Enterococcus faecalis; M. catarrhalis: Moraxella catarrhalis; B. subtilis: Bacillus subtilis; P. aeruginosa: Pseudomonas aeruginosa; P. falciparum: Plasmodium falciparum; L. donovani: Leishmania donovani; MRS: Methicillin resistant Staphylococcus

Table 2

Antibacterial and antifungal activities of pyridinium oximes

Entry	 16-34  35-61	IC_{50} (MIC) µg/mL					
		Antibacterial			Antifungal		
		S. aureus	*MRS*	*M. intracellulare*	*C. neoformans*	*C. glabrata*	*A. fumigatus*
21	R = 5-bromothiophen-2-yl, R_1 = 2-CHNOH, X = Cl	15.27	14.19	NA	na	>20	na
24	R = benzothiophen-3-yl, R_1 = 2-CHNOH, X = Cl	12.23 (20)	17.72 (20)	>20	na	na	na
25	R = benzothiophen-3-yl, R_1 = 3-CHNOH, X = Cl	na	na	na	na	na	>20
33	R = 3-methyl-isoxazol-5-yl, R_1 = 3-CHNOH, X = Br	na	na	na	>20	na	na
45	R_1 = 4-$CONH_2$, R_2 = 2-CHNOH, A = CH_2CH_2, X = Br	na	na	na	>20	na	na
46	R_1 = 4-$CONH_2$, R_2 = 3-CHNOH, A = CH_2, X = Br	na	na	na	>20	na	na
50	R_1 = R_2 = 2-CHNOH, A = thiophen-2,5-yl, X = Cl	na	na	na	>20	na	na
51	R_1 = R_2 = 3-CHNOH, A = thiophen-2,5-yl, X = Cl	na	na	na	>20	na	na
52	R_1 = R_2 = 4-CHNOH, A = thiophen-2,5-yl, X = Cl	na	na	na	>20	na	na
53	R_1 = 4-$CONH_2$, R_2 = 4-CHNOH, A = thiophen-2,5-yl, X = Cl	na	na	na	>20	na	na
58	R_1 = R_2 = 4-$CONH_2$, A = furan-2,5-yl, X = Br	na	na	na	>20	na	na
60	R_1 = R_2 = 3-CHNOH, A = isoxazol,3,5-yl, X = Br	na	na	na	>20	na	na

Entry		IC_{50} (MIC) µg/mL					
		Antibacterial			Antifungal		
		S. aureus	***MRS***	***M. intracellulare***	***C. neoformans***	***C. glabrata***	***A. fumigatus***
	 **16-34**						
	 **35-61**						
61	$R_1 = R_2$ = 4-CHNOH, A = isoxazol,3,5-yl, X = Br	na	na	na	>20	na	na
Ciprofloxacin		0.1 (0.5)	0.09 (0.25)	0.39 (0.5)	nt	nt	nt
Amphotericin B		nt	nt	nt	1.28 (2.5)	0.7 (1.25)	0.98 (2.5)

IC_{50}, the concentration that affords 50% inhibition of bacterial/ fungal growth; na, not active; nt, not tested; MIC (Minimum inhibitory concentration) is the lowest concentration (µg/ml) that allows no detectable growth; *None of the compound was active against *C. albicans*, *C. krusei*, *E. coli* and *P. aeruginosa* (data not shown).

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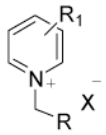
Table 3

Antimalarial and antileishmanial activities of pyridinium oximes

Entry	 16-34	Antimalarial		Antileishmanial		Cytotoxicity
		P. falciparum		*L. donovani*		Vero cells
		D6 Clone	W2 Clone			
		IC_{50} µg/mL		IC_{50} µg/mL	IC_{90} µg/mL	IC_{50} ng/mL
17	R = $CH_2CH_2CH_2Br$, R_1 = 4-$CONH_2$, X = Br	3.7 (S.I. > 1.3)	4.0 (S.I. > 1.2)	na	na	nc
21	R = 5-bromothiophen-2-yl, R_1 = 2-CHNOH, X = Cl	na	na	19	40	nc
24	R = benzothiophen-3-yl, R_1 = 2-CHNOH, X = Cl	na	na	18	40	nc
Chloroquine		0.015	0.12	nt	nt	nt
Artemisinin		0.010	0.0065	nt	nt	nt
Pentamidine		nt	nt	1.2	5	nt
Amphotericin B		nt	nt	0.2	0.4	nt

IC_{50}, the concentration that affords 50% inhibition of plasmodial / leishmanial growth; IC_{90}, the concentration that affords 90% inhibition of leishmanial growth; na, not active; nt, not tested; nc, Not cytotoxic; S.I., selectivity index = IC_{50} vero cells/IC_{50} *P. falciparum*.