

# Bifunctional Anti-Inflammatory and Anticholinergic Pro-Drugs as Potential Therapeutics for Sulfur Mustard-Induced Blisters

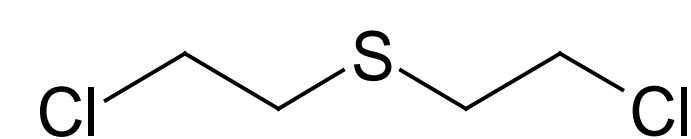


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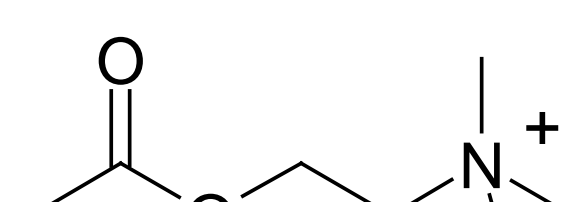
## Introduction

Sulfur mustard (2,2'-dichloroethyl sulfide, SM) is a chemical vesicant which alkylates DNA and is thus strongly mutagenic and carcinogenic. Additionally, SM induces severe skin, eye and lung blisters. Although this blistering agent has been used in chemical warfare since World War I, there is no effective treatment for sulfur mustard poisoning. Given the current world condition, the threat of SM use as a terrorist weapon is ever present. Much research has recently been done to try to understand the mechanism of toxicity of SM and thus design a potent and effective treatment.

Various studies have suggested that the synthesis of acetylcholinesterase (AChE), an enzyme which breaks down the neurotransmitter, acetylcholine (ACh), is increased following exposure to SM<sup>1</sup>. Also, inflammatory responses play a role in the dermal, respiratory and ocular damage associated with mustard exposure. Linking an anti-inflammatory moiety such as a non-steroidal anti-inflammatory drug (NSAID) with an AChE inhibitor in the same molecule should thus provide a dual benefit against SM<sup>2</sup>.



Structure of Sulfur Mustard (top) and SM-Induced Blisters (bottom)

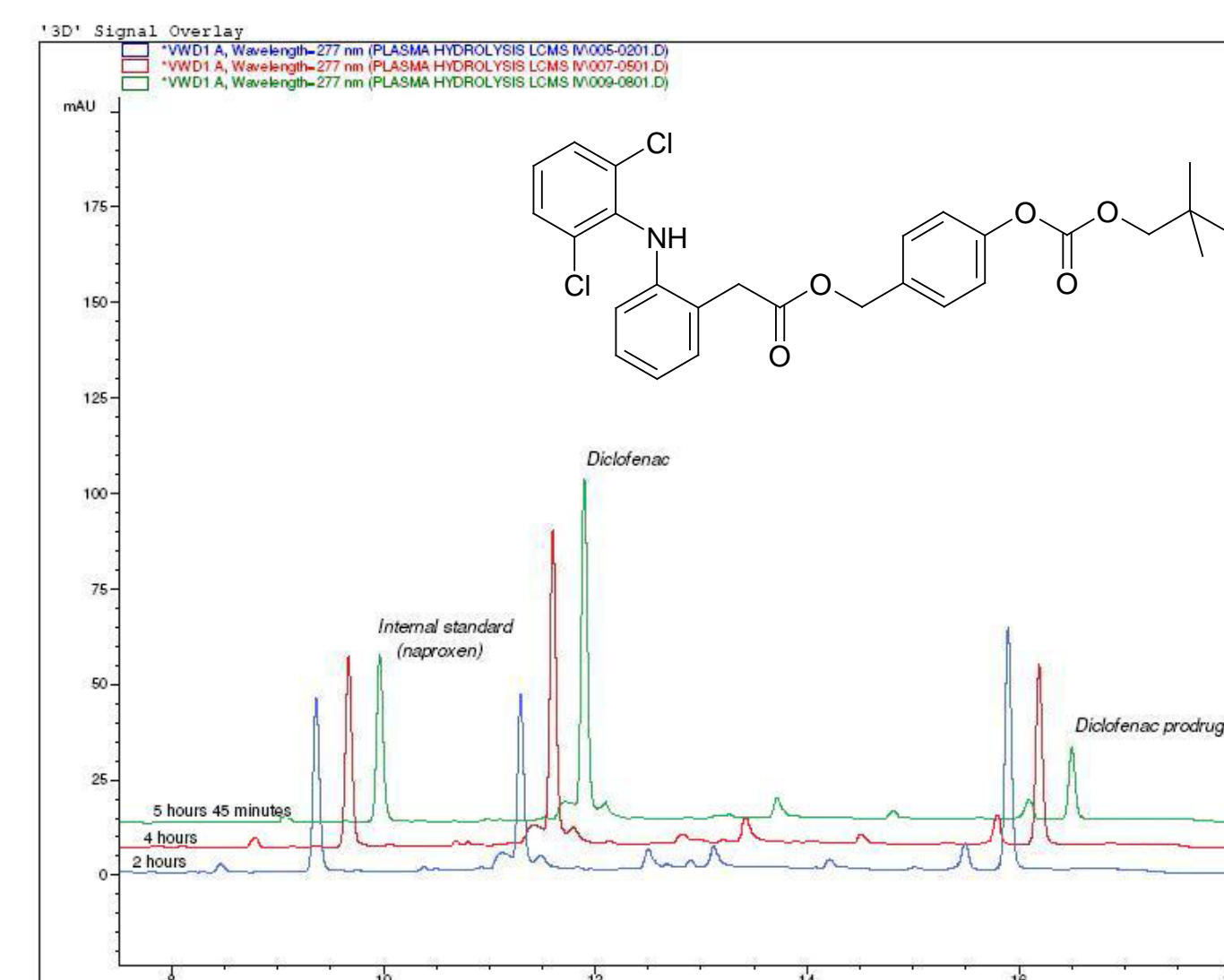


Acetylcholine Structure

## Dual-Action Pro-Drug

Almost all NSAIDs contain a carboxylic acid functionality which can irritate the gastrointestinal (GI) tract and cause other undesirable systemic effects. Masking the acidic portion of a particular NSAID by forming an ester linkage has been shown to both reduce GI side effects associated with chronic NSAID use and increase the drug's lipophilicity. Our research team has been synthesizing various esters of diclofenac, ibuprofen, naproxen and indomethacin. These drugs are considered **pro-drugs** of the parent NSAIDs which release the active ingredient over time upon enzymatic hydrolysis. Since these pro-drugs also have increased lipophilicity, they can be used in topical formulations which usually exhibit a localized therapeutic effect and less severe side effects<sup>3</sup>.

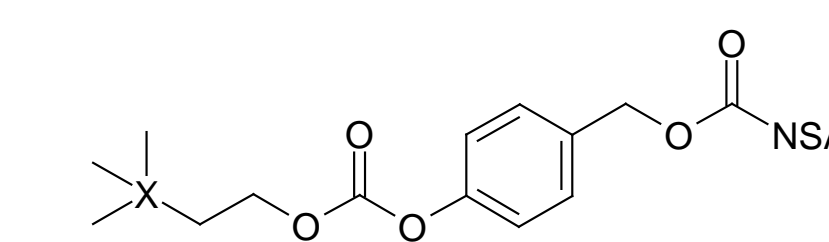
The stability of the diclofenac pro-drug (right) in human plasma was investigated using a previously developed method<sup>3a</sup>. Specifically, a 20  $\mu$ M solution of pro-drug in 2% DMSO and 80% plasma (diluted with 0.16 M pH 7.4 phosphate buffer) was incubated at 37°C for several hours. Aliquots were withdrawn and analyzed at suitable time intervals.



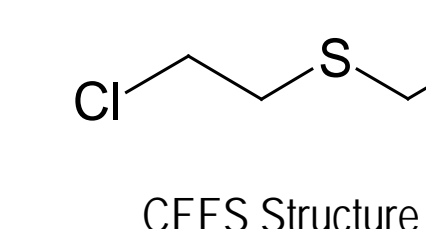
The data show a decrease in the amount of pro-drug and a simultaneous increase of diclofenac free acid over time. There seems to be a gradual release of the diclofenac parent drug as the pro-drug is metabolized in plasma.

## CEES Mouse Ear Vesicant Model

CEES (2-chloroethyl ethyl sulfide), a less severe sulfur mustard analogue, was applied topically to the ears of female CD-1 mice (24-25 days old) in 20  $\mu$ L of  $\text{CH}_2\text{Cl}_2$  or acetone to generate an inflammatory response. Edema was measured by determining the increase in the wet weight of ear punch biopsies. To evaluate each drug, ears were pretreated with 20  $\mu$ L of vehicle control or 20  $\mu$ L of test compounds 20 min prior to treatment with CEES. Five hours later, all mice were sacrificed. The ear punches (6 mm in diameter) were taken and weighed. The data were analyzed as percent inhibition of vesicant-induced edema.



NSAID	X	% CEES Inhibition
Indomethacin	C	54.1
Indomethacin	Si	1.04
parent drug	----	46
Ibuprofen	C	51.2
Ibuprofen	Si	6.04
Diclofenac	C	98
Diclofenac	----	17
parent drug	----	17
(S)-Naproxen	C	12.29

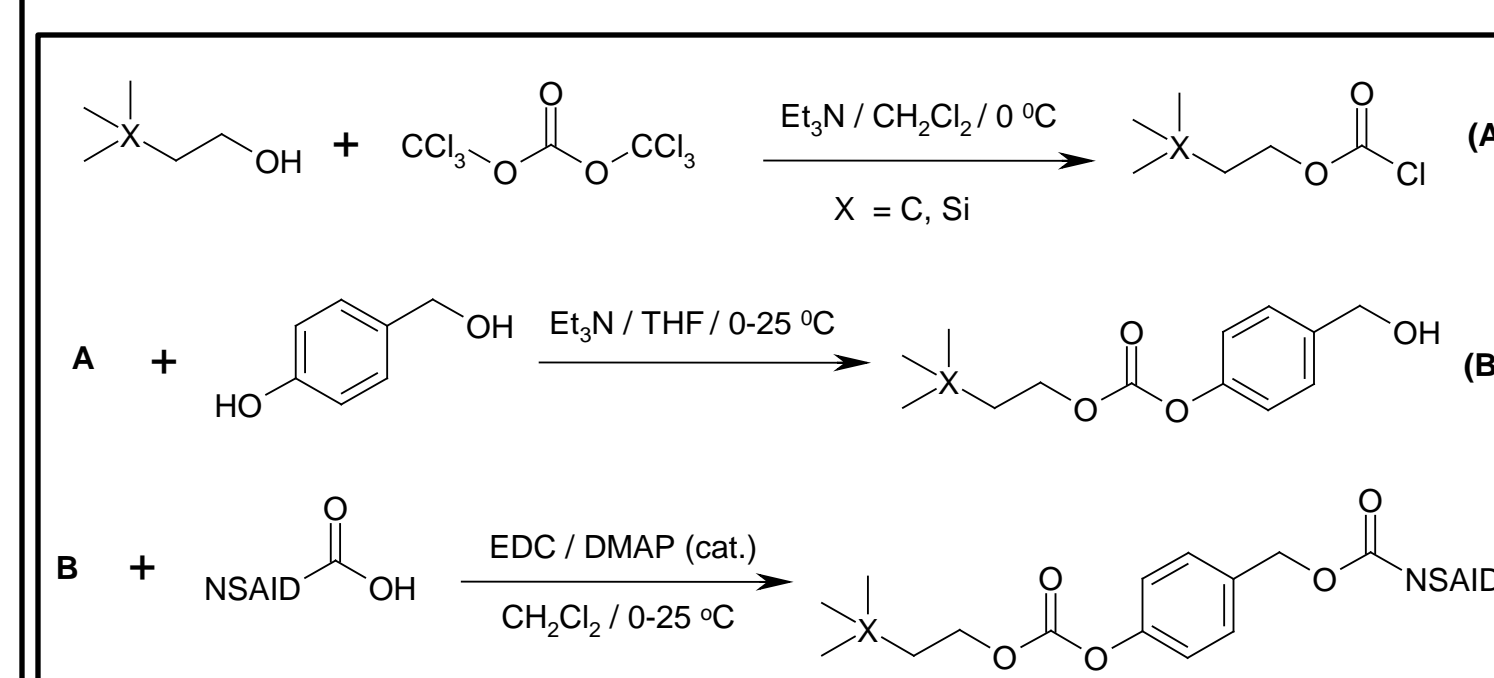
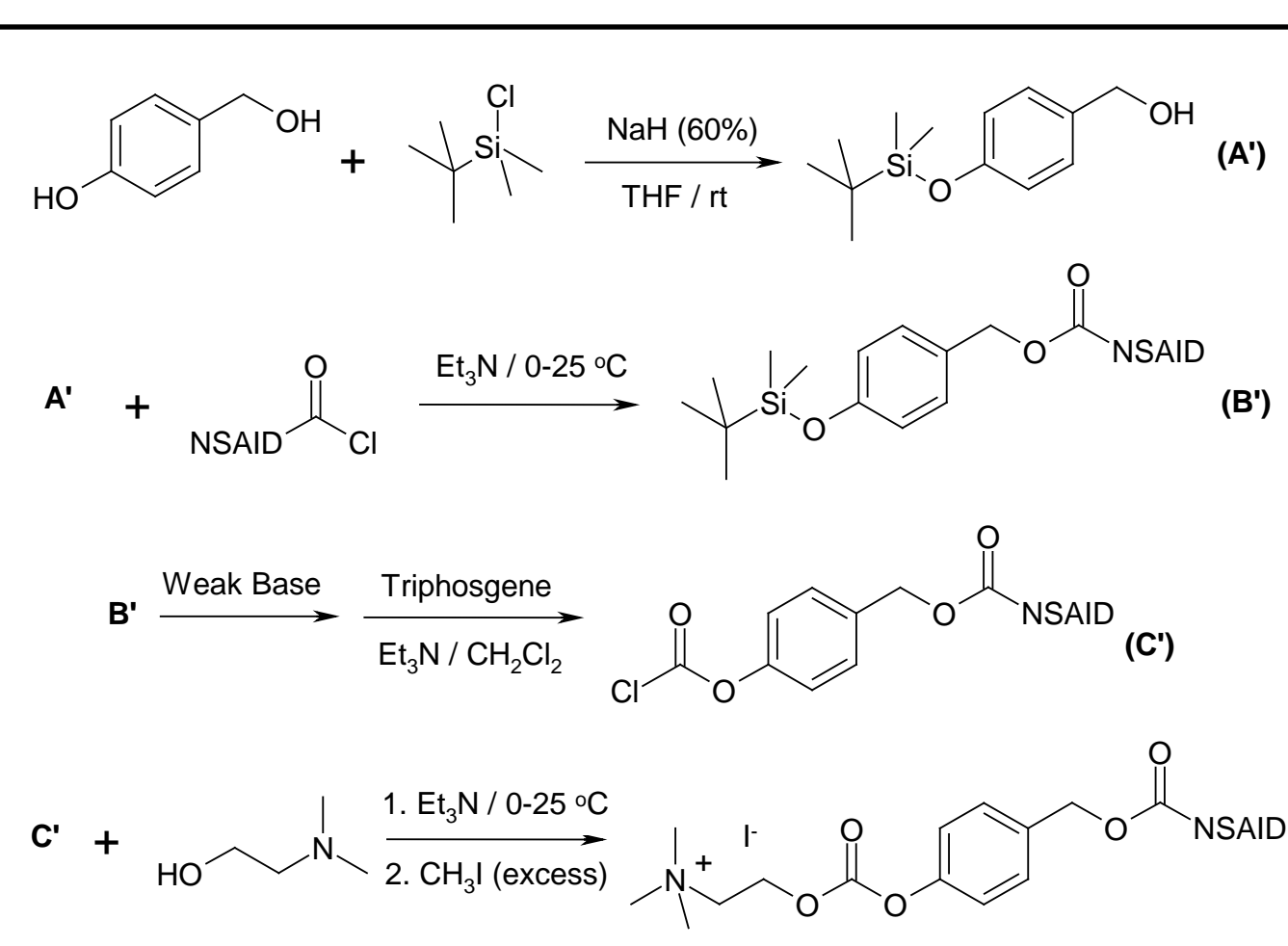
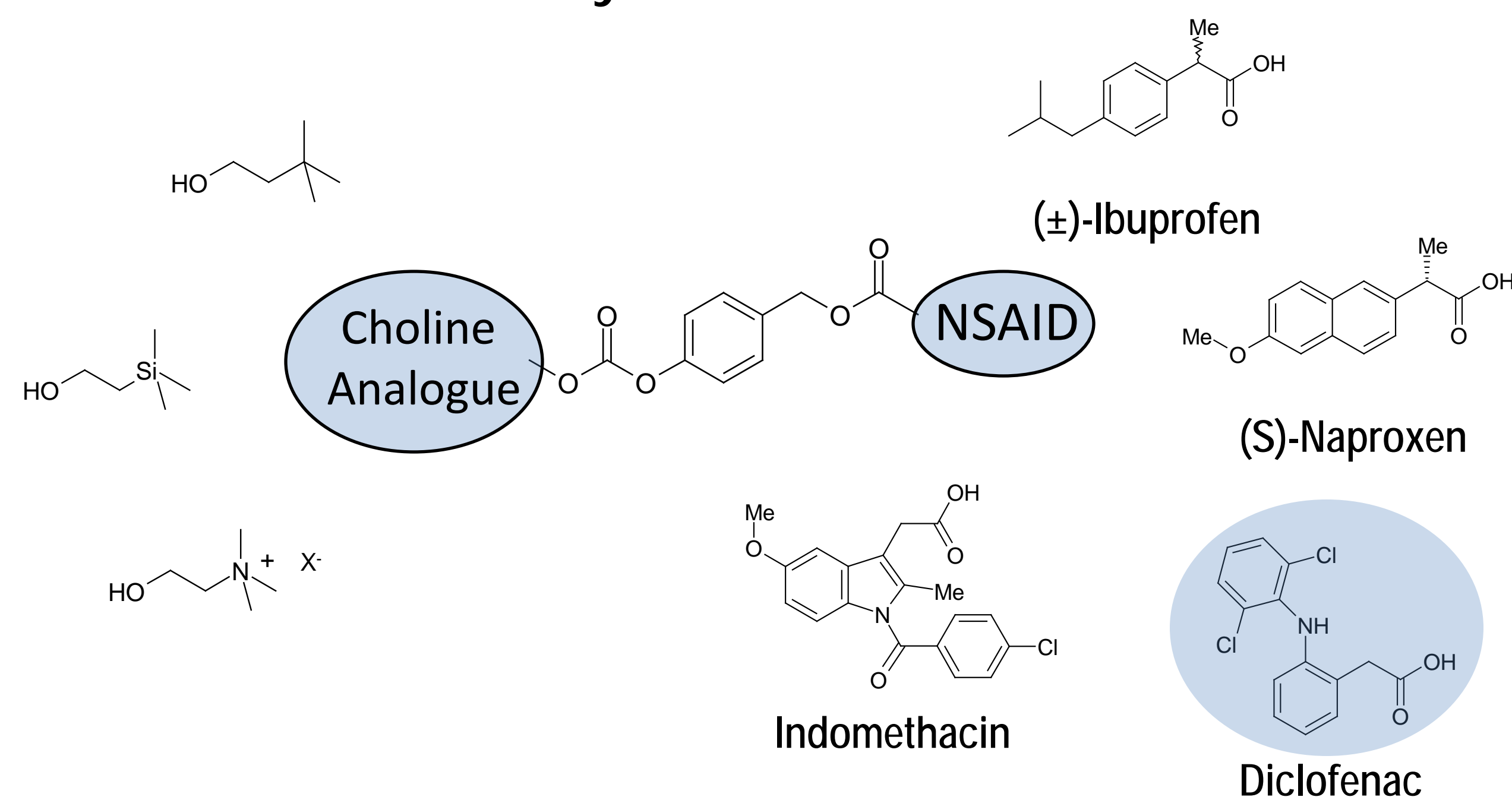


CEES Structure



The photos above represent the effect of CEES on a mouse ear over time.

## Synthesis

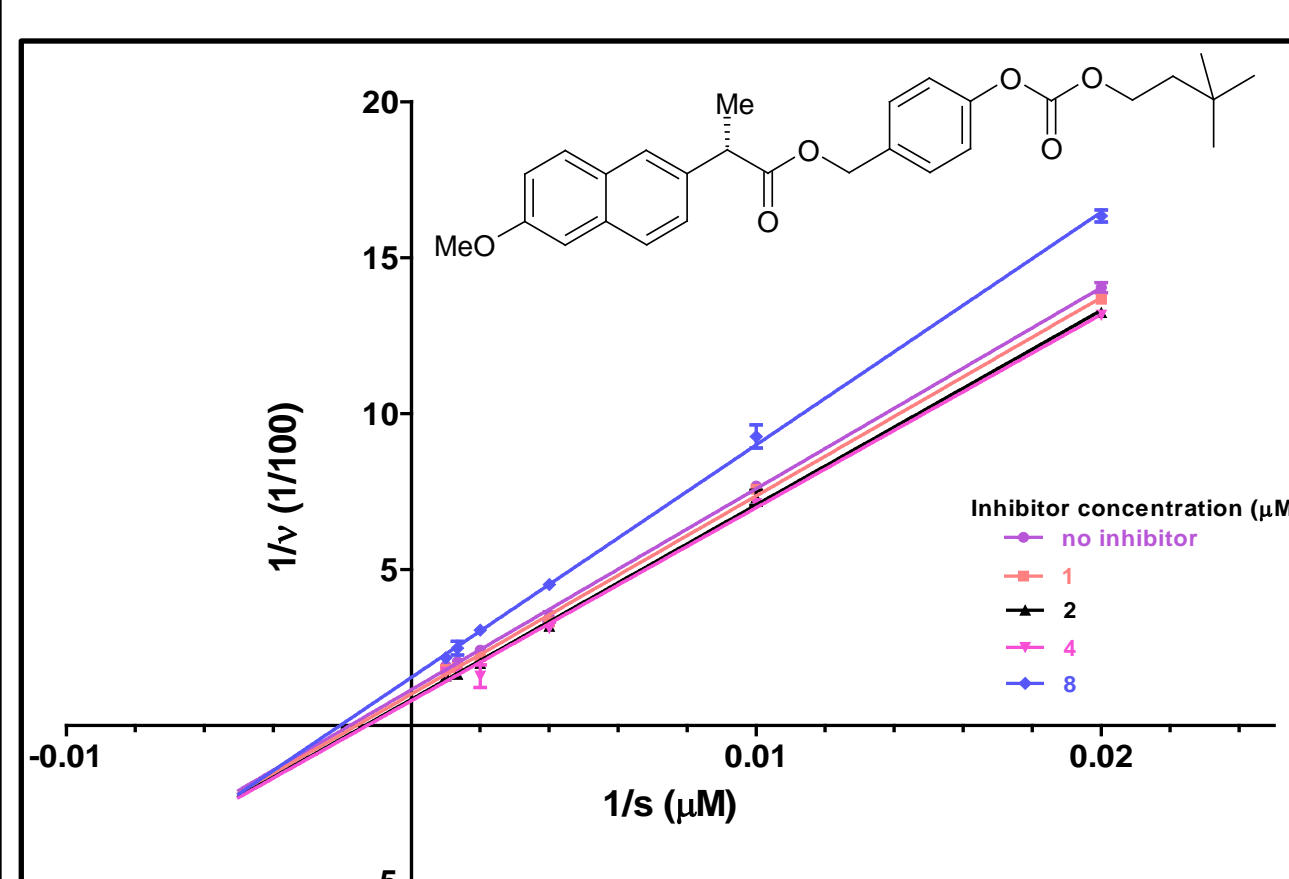


## AChE Inhibition

Cholinesterase inhibition was assayed spectrophotometrically at 412 nm according to Ellman's method (*Biochem. Pharmacol.* 1961, vol 7, 88-95): 200  $\mu$ L of 0.5 mM DTNB in 100 mM sodium phosphate buffer (pH 8), 30  $\mu$ L of inhibitor stock solution prepared in methanol, 50  $\mu$ L of 3 mM AChI and 20  $\mu$ L of 1.25 u/mL AChE (Type V-S from *Electrophorus electricus*), prepared respectively in phosphate buffer 100 mM pH 8 and 20 mM pH 7. Immediately after the enzyme was added, the signal was measured at 30 s intervals over 5 min at 25°C.  $\text{IC}_{50}$  values were obtained from a minimum of eight concentrations in duplicate and by fitting the experimental data with a dose-response curve using Prism Version 5.00, GraphPad Software, San Diego, CA. Refer to data table (right) and corresponding dose-response curve (below).

NSAID	X	$\text{IC}_{50}$ ( $\mu$ M)
Indomethacin	C	2.29
Indomethacin	Si	0.72
Ibuprofen	C	1.93
Ibuprofen	Si	1.19
Ibuprofen	N <sup>+</sup>	77.7
Diclofenac	C	0.51
Diclofenac	Si	1.36
(S)-Naproxen	C	1.74
(S)-Naproxen	Si	0.83
Tacrine, HCl	--	0.045*

\*Tacrine is a potent reversible AChE inhibitor.  $\text{IC}_{50}$  value from *J. Med. Chem.* 2008, 51, 7666.



The Lineweaver-Burk plot (left) was generated by plotting the reciprocal of the velocity (1/v) against the reciprocal of substrate concentration (1/s). Interception in quadrant III reflects **reversible inhibition**. A fixed amount of enzyme (0.025 u) and varying amount of both substrate (1000 to 50  $\mu$ M) and inhibitor (1 to 12  $\mu$ M) were used. Additionally, when AChE enzyme activity was assayed after 30 min incubation with 52  $\mu$ M inhibitor and subsequent purification by Sephadex gel, close to 100 % enzyme activity was recovered. Tacrine hydrochloride was used as a control and resulted in 95 % AChE recovery. AChE activity was determined by Ellman's method as discussed above.

## Conclusions

The most potent AChE inhibitor (diclofenac analogue with quaternary carbon) also shows the best protection against CEES.

These bifunctional molecules seem to act as pro-drugs which may induce more localized therapeutic effects with less severe GI side effects.

The lipophilic nature of these compounds makes them suitable candidates for preventative topical formulations to treat sulfur mustard poisoning.

## Future Work

Investigate the hydrolysis of pro-drugs as they pass the skin.

Test simple esters of bifunctionals to determine if the "linker" is necessary for potent anti-inflammatory and anti-cholinergic activity.

Further expand class of dual-action drugs and optimize current synthetic methods. *\*Ultimately support the link between increased AChE synthesis and inflammation, which remains a topic of debate in the literature.*

## References

- Brenner *et al. Neuropharm.* 2006, 50, 540-547.
- Amitai *et al. J. Appl. Toxicol.* 2006, 26, 81-87.
- (a) Rautio *et al. J. Pharm. Sci.* 1998, 87 (12), 1622-1628; (b) Khan *et al. J. Pharm. Sci.* 1994, 83, 644-648.