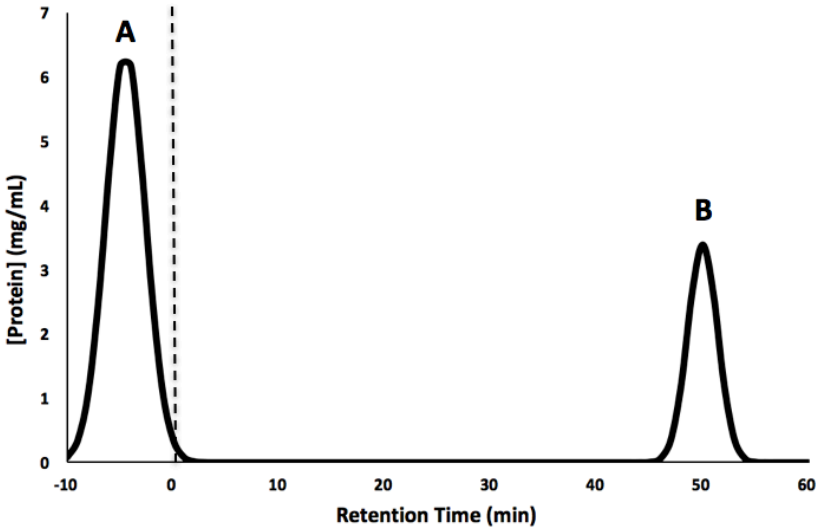
Name: _____ Exam 2

Quoted text and questions for this exam are from: Nys *et al.* (2016). Allosteric binding site in a Cys-loop receptor ligand-binding domain unveiled in the crystal structure of ELIC in complex with chlorpromazine. *PNAS* [Published online] E6696-E6703.

The authors describe the protocol to purify *Erwinia* Ligand-gated Ion Channels (ELIC) in the following text:

> "ELIC was expressed and purified as previously described (36), with minor modifications. ELIC was cloned into pET-11a (Novagen) and N-terminally fused to maltose-binding protein (MBP) with a 3CV protease cleavage site for removal of MBP. The MBP–ELIC fusion protein was expressed in the C43 Escherichia coli strain. Membranes were solubilized with 2% (wt/vol) anagrade n-undecyl-β-D-maltoside (Anatrace), and the soluble fraction was purified by affinity chromatography on amylose resin (New England Biolabs). Affinity-bound protein was cleaved by 3CV protease and further purified on a Superdex 200 10/300 GL (GE Healthcare) column equilibrated with buffer containing 10 mM Na-phosphate (pH 8.0), 150 mM NaCl, and 0.15% n-undecyl-β-D-maltoside."

1. Imagine that the following graph depicts the result of the affinity column. The solubilized cell product was applied to the column 10 minutes before (-10 min) the 3CV protease was applied to the column at the time of zero (0 min). Which peak (*A* or *B*) should contain ELIC?



2. Imagine that the following graph depicts the results of the Superdex 200 10/300 GL column (a size-exclusive resin). Five proteins were loaded onto the column each with the following characteristics:

<u>Glutamate dehydrogenase</u>
447 amino acids (49 kDa)
pI 6.0
54 basic residues
50 acidic residues

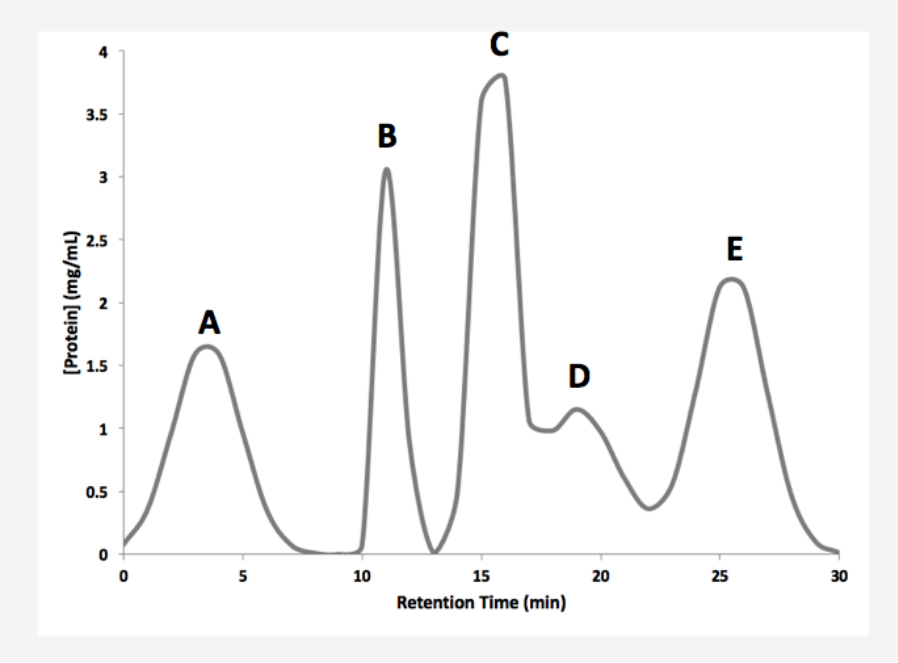
<u>ELIC</u>
1605 amino acids (184 kDa)
pI 5.3
155 basic residues
165 acidic residues

<u>Lac repressor</u>
363 amino acids (39 kDa)
pI 7.5
37 basic residues
32 acidic residues

<u>Beta-galactosidase</u>
1024 amino acids (116 kDa)
pI 5.5
120 basic residues
126 acidic residues

<u>RNA polymerase omega subunit</u>
91 amino acids (10 kDa)
pI 4.5
13 basic residues
17 acid residues

Which peak (*A; B; C; D; or E*) should contain ELIC?



3. The authors equilibrate their protein in 10 mM Na-phosphate (pH 8.0), 150 mM NaCl, and 0.15% n-undecyl-β-D-maltoside. Calculate what you would add to prepare 1 L of a stock solution that is ten-fold concentrated:

1L of Stock with:

100 mM Na-phosphate (pH 8.0; pKa 7.0)
1.5 M NaCl
1.5 % (30 mM) n-undecyl-β-D-maltoside

- Na phosphate monobasic dihydrate (NaH_2PO_4 $2H_2O$; acidic form; MW 156.01 g/mole)
- Na phosphate dibasic heptahydrate (Na_2HPO_4 $7H_2O$; basic form; MW 268.07 g/mole)
- Sodium chloride (NaCl; MW 58.44 g/mole)
- n-undecyl-β-D-maltoside ($C_{23}H_{44}0_{11}$; 496.59 g/mole)

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After finding that CPZ did not bind in the channel of ELIC in their x-ray crystal structure, the authors performed a number of mutations in the protein region where they suspected that CPZ actually bound. The particular mutation of introducing a Cys at each position allowed the authors to react the protein with a modified CPZ (MTS-PZ) that could result in a covalent crosslink between the Cys at the indicated position and the MTS-PZ. The results of the Cys mutations in the absence of CPZ or MTS-PZ were summarized in Table 1:

Table 1. Summary of the functional characterization of WT and mutant ELIC: GABA EC_{50} and n_H and $I_{max} \pm$ SEM

ELIC construct	EC_{50}, mM	n_H	I_{max}, μA	n
Wild-type ELIC	21 ± 1.0	2.1 ± 0.20	21 ± 2.4	4
Cys-less ELIC	23 ± 3.2	2.3 ± 0.70	16 ± 1.9	4
I20C	62 ± 8.0*	2.0 ± 0.40	3.8 ± 0.70	5
N21C	30 ± 5.3	2.1 ± 1.0	1.3 ± 0.10	3
I23C	24 ± 3.6	2.3 ± 0.80	4.9 ± 2.90	2–3
F126C	14 ± 0.5*	2.2 ± 0.10	6.9 ± 3.60	3–5
V147C	41 ± 12.2	1.7 ± 0.90	21 ± 5.8	3
T149C	16 ± 1.1*	2.4 ± 0.30	31 ± 3.1	3
E150C	20 ± 7.8	2.0 ± 1.30	6.2 ± 1.40	3
E155C	20 ± 2.7	2.7 ± 0.70	11 ± 1.9	3–5
D158C	8.5 ± 2.00*	3.0 ± 2.30	6.6 ± 1.30	2–4
W160C	3.5 ± 0.30*	3.1 ± 0.80	0.20 ± 0.07	3–4
I162C	22 ± 5.5	2.3 ± 1.10	6.6 ± 3.20	3

*$P < 0.05$, significantly different from Cys-less ELIC, Student's t test.

The results of the inhibition by covalent crosslinking of MTS-PZ were summarized in Figure 4 (panel D):

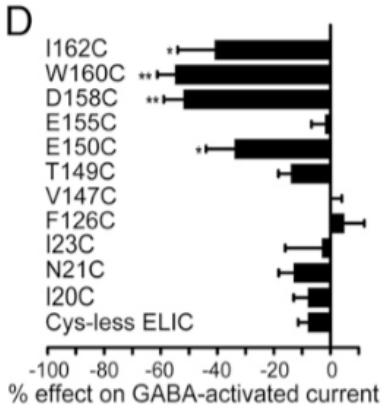
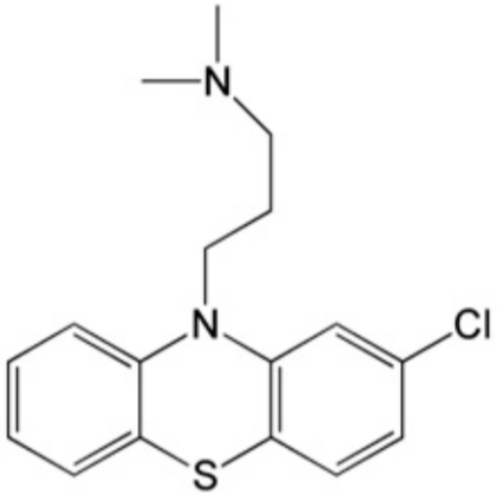


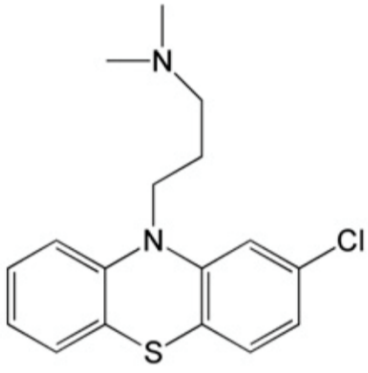
Fig. 4. Cysteine-scanning mutagenesis of the β8–β9 loop in ELIC. (*A*) Electrophysiological recordings of Cys-less ELIC in response to repetitive pulses of GABA at the EC_{50} (=20 mM) and application of 200 μM of a thiol-reactive CPZ derivative termed MTS-PZ. (*B*) Example traces of a Cys mutant, E155C, showing no effect of MTS-PZ. (*C*) Example traces of a Cys mutant, D158C, showing an inhibitory effect of MTS-PZ. (*D*) Summary of MTS-PZ–mediated channel inhibition on the different Cys mutants. Data represent the mean ± SEM of three to five experiments. *$P < 0.05$, significantly different from Cys-less ELIC, Student's t test; **$P < 0.01$, significantly different from Cys-less ELIC, Student's t test.

4. Which residue positions had a significant effect in ***both*** the Cys mutation and the MTS-PZ crosslinking studies?

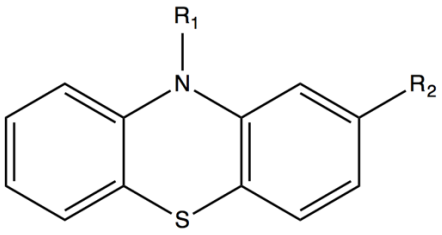
5. Use PyMol to locate where the residues of ELIC (PDB ID: 5LG3) that you found for the previous question (#4) interact with CPZ. Indicate the positions on the CPZ structure below with chemical structures. Identify the type of intermolecular interaction for each.



6. If you were going to begin organic derivatization studies on CPZ to try to make a better inhibitor, what portion or portions of CPZ would you suggest to begin changing? Circle your answer on the CPZ molecule below:



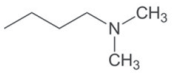
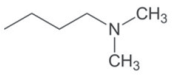
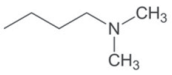
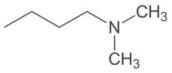
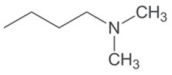
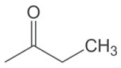
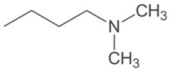
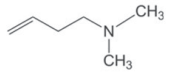
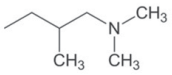
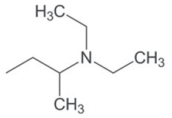
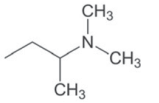
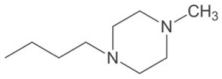
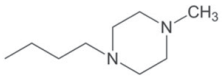
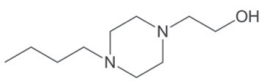
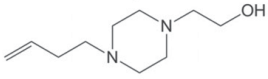
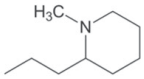
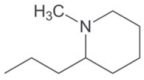
The authors determined the IC_{50} for a number of CPZ derivatives, which are summarized in their Table S2 (see next attached page). Significant differences are indicated by *.



7. Explain whether your suggestions from the previous question (#6) turned out to be correct. The ring portion of CPZ is not as easily derivatized as the R_1 and R_2 positions. Limit your discussion to the R_1 and R_2 portions.

8. Do the derivatives with significantly increased binding affinity predominately add nonpolar, hydrophobic surface or polar, hydrophilic surface to the inhibitor? Is this consistent with your PyMol image if the molecule must make stronger intermolecular interactions to lower the IC_{50}?

Table S2. Pharmacological properties of phenothiazine analogs on ELIC

Compound	R_1	R_2	IC_{50} ± SEM, μM	n_H ± SEM	*n*
CPZ		—Cl	158 ± 37.1	−1.6 ± 0.50	3–13
Acepromazine			97.7 ± 29.65	−0.89 ± 0.205	4
BrPZ		—Br	166 ± 29.4	−2.6 ± 0.95	4–5
Promazine		—H	240 ± 38.8	−0.90 ± 0.677	5–6
Propionylpromazine			130 ± 43.2	−1.4 ± 0.56	2–4
Triflupromazine		—CF_3	43.3 ± 99.40	−0.87 ± 0.649	4
Chlorprothixene		—Cl	N.S.		
Cyamemazine		—CN	33.9 ± 3.87*	−2.5 ± 0.61	1–4
Ethopromazine		—H	N.S.		
Promethazine		—H	58.4 ± 6.99*	−2.1 ± 0.62	3
Prochlorperazine		—Cl	27.5 ± 3.34*	−1.0 ± 0.11	3
Trifluoperazine		—CF_3	58.0 ± 11.4*	−2.0 ± 0.87	1–7
Perphenazine		—Cl	N.S.		
Flupentixol		—CF_3	45.5 ± 2.15*	−1.6 ± 0.13	3
Mesoridazine			66.7 ± 1.93*	−2.3 ± 0.17	5
Thioridazine		—SCH_3	154 ± 8.4	−1.4 ± 0.08	3

N.S., not soluble after dilution in water of a 10-mM stock solution in DMSO.