Supplementary Information for

Functional Selection of Protease Inhibitory Antibodies

Tyler Lopez[a], Zahid Mustafa[a], Chuan Chen[a], Ki Baek Lee[a], Aaron Ramirez[a], Chris Benitez[a], Xin Luo[b], Ru-Rong Ji[b], Xin Ge[a1]

[a]Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521; [b]Center for Translational Pain Medicine, Department of Anesthesiology, Duke University Medical Center, Durham, NC 27710

[1]To whom correspondence may be addressed: xge@engr.ucr.edu

CONTENT:

www.pnas.org/cgi/doi/10.1073/pnas.1903330116

SI Materials and Methods

Development of protease cleavage reporters. Plasmid carrying β-lactamase TEM-1 gene was PCR amplified to introduce unique *Xba*I and *Nco*I recognition sites between G196 and E197 of TEM-1. The PCR product was ligated with 5' phosphorylated oligonucleotide assembled adapters encoding protease specific cleavable peptide sequences (**Table S1**) flanked by serine-glycine linkers (GSG[peptide]SGG) to obtain genes of modified TEM-1s. Genes encoding extracellular or catalytic domains of human MMP-9 (residue 107-443 without fibronectin domains), human MMP-14 (residue 114-320), human β-secretase 1 (BACE1, residue 46-457), *Aspergillus fumigatus* autophagic serine protease 2 (Alp2, residue 136-495), and human cathepsin B (residue 66-339) without their associated propeptide sequences were PCR assembled and cloned into *Sfi*I sites on pMopac16 carrying a p15A origin and a pelB leader peptide to obtain their periplasmic expression plasmids (1). The modified TEM-1 genes were then sub-cloned into these protease expression plasmids using *Nsi*I and *Nhe*I sites to generate reporter plasmids (**Fig 1A**). From these reporter plasmids, protease genes were then removed by PCR amplifying the TEM-1 gene regions followed by self-ligation to obtain plasmids carrying modified TEM-1 genes without protease genes. All cloned plasmids were confirmed by DNA sequencing. β-lactam ring hydrolysis activities of modified TEM-1s in the absence or presence of associated proteases were tested by culturing transformed *E. coli* BL21 cells on 2×YT agar plates containing 34 μg/mL chloramphenicol, 50 μg/mL kanamycin, 0-0.1 mM IPTG, 0-2% glucose, and 0-1000 μg/mL ampicillin at 30 ºC for 16 hours. Colony numbers were determined by serial dilutions and survival curves were plotted to identify the optimal conditions of inhibitor selection for each protease target.

Selection of protease inhibitory antibodies. Fab library genes containing regular (2) or ultra-long CDR-H3s (3) were PCR amplified and cloned into pHPK (kanR, pBR322 ori, phoA promoter, and STII leader). Constructed library plasmids pHPK-Fab were transformed into *E. coli* Jude-I electrocompetent cells for amplification. Randomly picked colonies were sequenced for library quality and diversity tests. Electrocompetent cells of BL21 harboring the reporter plasmid for individual protease were transformed with 100 μg library pHPK-Fab. Transformed cells were cultured on 2×YT agar plates of pre-determined selection conditions specific for each protease (**Table S1**). Small aliquots of transformed cells were also serially diluted and cultured on 2×YT agar plates supplemented with 34 μg/mL chloramphenicol and 50 μg/mL kanamycin for library size determination. Colonies surviving the initial selection were individually inoculated in the 2×YT selection media with a higher ampicillin concentration for secondary screening. Well-grown clones were selected for Fab plasmid extraction and V_H and V_L DNA sequencing.

Production of antibodies and proteases. Genes of isolated Fabs were sub-cloned and transformed into BL21 cells for periplasmic production by culturing in 2×YT media at 30 ºC for 12 hours. Fabs with a hexahistidine tag at the C-terminal of V_H were purified using Ni-NTA agarose (Qiagen) from periplasmic fractions prepared by lysozyme and osmotic shock (4). Associated IgGs were produced in HEK293F (ThermoFisher Scientific) as previously described (5). Purified Fabs and IgGs were dialyzed at 4 ºC against the following assay buffers: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.4 mM $ZnCl_2$ for cdMMP-9; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$ for cdMMP-14; PBS pH 7.5 for Alp2 and cathepsin B; and 20 mM HEPES, 125 mM NaCl pH 5.0 for BACE-1. Dialyzed antibody samples were concentrated by 10 kDa MWCO ultrafiltration (Amicon), and their purity and concentration were determined by SDS-PAGE and UV spectrophotometer (BioTek). C-terminal hexahistidine tagged cdMMP-9, cdMMP-12, cdMMP-14, Alp2, cathepsin B, and cathepsin K were produced in their active format in the periplasmic space of *E. coli* without refolding or activation (1) and purified using Ni-NTA agarose (Qiagen). MMP-2 was purchased from AnaSpec Inc. BACE1-Fc fusion was produced by HEK293F using pcDNA-intron-SPL-BACE1-Fc-WPRE containing human IgG1 heavy chain with the associated signal peptides and Woodchuck hepatitis virus posttranscriptional regulatory elements to enhance the expression. Cultured media was clarified by centrifugation and 0.45 μm filtration, and BACE1-Fc was purified by protein A affinity chromatography (GenScript).

Biochemical characterizations of isolated antibodies. Binding kinetics of produced antibodies towards associated protease targets were analyzed by using biolayer interferometry (ForteBio). For Fabs, biotinylated proteases were immobilized on streptavidin biosensors, and Fab binding to the sensors in absence of protease was monitored as backgrounds. For IgGs, protein A sensors were used and protease bindings without IgG were checked as backgrounds. k_{on} and k_{off} were determined for K_D calculations. Competitive ELISA of Fabs on immobilized cdMMP-9/-14 in the presence of 1 nM-1 μM nTIMP-2 was also tested. Fab *in vitro* stability was tested by incubating 1 μM Fab with 1 μM of the respective protease in the assay buffer for 12 hours and the samples were analyzed by SDS-PAGE.

For inhibition tests, 1 μM Fabs were 2-fold serially diluted into protease specific assay buffer and incubated with 1-10 nM proteases for 30 min at room temperature. The kinetic measurements were started with the addition of 1 μM following FRET peptide substrates: M-2350 (Mca-KPLGL-Dpa(Dnp)-AK-NH_2, Bachem) for MMP-9/14, M-2420 (Mca-SEVNLDAEFK(Dnp)-OH, Bachem) for BACE-1, Mca-KLRSSKQK(Dnp) (Biomatik) for Alp2, and M-2595 (Abz-GIVRAK(Dnp)-OH, Bachem) for cathepsin B. The generated fluorescence signals were monitored with excitation and emission wavelengths at 325 and 392 nm (except M-2595 at 320/420 nm) using a fluorescence plate reader (BioTek). Inhibition percentages at given concentrations were calculated by comparing the initial reaction rates in the presence or absence of inhibitor. IC_{50} was determined as the concentration that achieved 50% inhibition, and K_I values were calculated using $K_I = IC_{50}/(S/K_m+1)$. V_{max} and K_m at various Fab concentrations were measured to determine the inhibition type. FRET inhibition assays were also used for selectivity tests of isolated Fabs with the relevant proteases.

For inhibition tests on macromolecular substrates, 1 μM cdMMP-9 was incubated with 300 μg/mL rat collagen I (Corning) with or without 1 μM Fab L13 in MMP-9 assay buffer at 37 ºC for 24 h. Samples were taken hourly and analyzed by SDS-PAGE under non-reducing conditions. For amyloid precursor protein (APP) degradation studies, $APP_{571-696}$ was cloned to the C-terminal of maltose binding protein (MBP) for *E. coli* expression and purified using amylose resin (NEB). 5 μM purified MBP-APP was incubated with 1 μM BACE1-Fc in the absence or presence of 1 μM IgG in BACE-1 assay buffer at 37 ºC for 24 h. In addition, $APP_{571-696}$ was cloned to a pcDNA plasmid carrying a SPE leader for secretion expression. Transfected HEK293F cells were cultured in Expi293 media for 72 hrs with the presence of 14-800 nM purified IgG. The supernatants were clarified by centrifugation and 0.45 μm filtration and produced $A\beta_{1-40}$ was quantified by ELISA (Abcam). For Alp2, 10 μg/mL FITC-conjugated type I collagen (AnaSpec) was incubated with 2 μM Alp2 in PBS at room temperature in the presence or absence of 10 μM Fab for 18 h. The reaction solution was centrifuged at 3,000 g for 10 min and the fluorescence of the supernatant was measured at Ex/Em=490/520 nm.

Biological functions of anti-MMP9 IgG L13: Neuropathic pain measurement. Wild-type CD1 mice (male and female, 8-10 wks, Charles River Laboratories) were housed at Duke vivarium animal facility, and all animal experiment protocols were approved. To produce chemotherapy-associated neuropathic pain, paclitaxel (PTX, 2 mg/kg, i.p.) was injected at day 0, 2, 4, and 6 (6). Intrathecal injection was performed as described previously (7), mice were anesthetized with isoflurane and a spinal cord puncture was performed between the L5 and L6 level to deliver drugs (10 μL) using a 30G needle. All behavioral tests were performed in boxes on an elevated metal mesh floor under stable room temperature and humidity. Mice were habituated to the environment for at least 2 days before the experiments. To assess mechanical allodynia, the plantar surface of the left hind-paw was stimulated using a series of von Frey fibers with logarithmically increasing stiffness (0.02-2.56 gram, Stoelting), presented perpendicularly to the central plantar surface. 50% paw withdrawal threshold was determined following Dixon's up-down method. The frequency response was measured by stimulating the hind-paw with a 0.4 gram von Frey hair for ten times and the percentage withdrawal response was calculated as frequency. All the behavioral tests were performed in a blinded manner.

SI References

1. Nam DH, Ge X. Direct production of functional matrix metalloproteinase--14 without refolding or activation and its application for in vitro inhibition assays. *Biotechnol Bioeng.* **113**, 717–723 (2016).
2. Persson H, et al. CDR-H3 Diversity is not Required for Antigen Recognition by Synthetic Antibodies. *J Mol Biol.* **425**, 803–811 (2013).
3. Nam DH, Ge X. Generation of Highly Selective MMP Antibody Inhibitors. *Methods Mol Biol.* **1731**, 307–324 (2018).
4. Rodriguez C, Nam DH, Kruchowy E, Ge X. Efficient Antibody Assembly in *E. coli* Periplasm by Disulfide Bond Folding Factor Co-expression and Culture Optimization. *Appl Biochem Biotechnol.* **183**, 520–529 (2017).
5. Chen KE, et al. Use of a Novel Camelid-Inspired Human Antibody Demonstrates the Importance of MMP-14 to Cancer Stem Cell Function in the Metastatic Process. *Oncotarget.* **9**, 29431–29444 (2018).
6. Chen G, Park CK, Xie RG, Ji RR. Intrathecal Bone Marrow Stromal Cells Inhibit Neuropathic Pain via TGF-β Secretion. *J Clin Invest.* **125**, 3226–3240 (2015).
7. Xu ZZ, et al. Inhibition of Mechanical Allodynia in Neuropathic Pain by TLR5-mediated A-fiber Blockade. *Nat Med.* **21**, 1326–1331 (2015).

Table S1. Conditions and results of selections for protease inhibitory antibodies

Protease	**cdMMP-9**		**cdMMP-14**	**BACE1**		**Alp2**	**cathepsin B**
Peptide insert	RLPLGI	SGRIGFLRTA	SGRIGFLRTA	EISEVKMDAEY		KLRSSKQ	KLHFSKQ
Library							
CDR-H3 length (aa)	23, 25, 27	23, 25, 27	23, 25, 27	23, 25, 27	5-21	5-21, 23, 25, 27	5-21
Size	6.2×10^8	4.1×10^8	8.6×10^8	7.1×10^8	1.8×10^8	2.9×10^8 *	1.5×10^8
Initial selection							
[Amp] (μg/mL)	300	300	200	300	300	300	300
[IPTG] (mM)	0.1	0.1	0	0.1	0.1	0.1	0.1
[glucose] (%)	0	0	2	0	0	0	0
Temp (°C)	30	30	30	30	30	30	30
# of clones remaining	22	37	190	24	87	43	122
Secondary screening							
[Amp] (μg/mL)	400	400	300	400	400	500	400
[IPTG] (mM)	0.1	0.1	0	0.1	0.1	0.1	0.1
[glucose] (%)	0	0	0	0	0	0	0
Temp (°C)	30	30	30	30	30	30	30
# of clones remaining	13	15	161	21	52	29	7 †
Sequenced	13	7	40	8	5	10	7
Unique correct sequences	13	7	38	6	5	8	5
Fabs produced	8	5	6	6	5	8	3
Binders	8	5	6	6	5	8	3
Inhibitors	8	3	6	6	5	6	3

Note:

* Long CDR library (CDR-H3 length = 23, 25 or 27) and normal CDR library (CDR-H3 length = 5-21) were combined for selection.

† Only 10 clones were randomly picked for the secondary screening.

Table S2. Statistical analysis for inhibitory antibodies shown in Table 1

Target	Fab	Binding affinity *			Inhibition potency †				
		k_{on} ± S.E. (1/Ms)	k_{off} ± S.E. (1/s)	K_D ± S.E. (nM)	a ± S.E.	b ± S.E.	I_0 ± S.E.	IC_{50} ± S.E. (nM)	K_I ± S.E. (nM)
MMP-9	H4	$(4.62\pm0.42)\times10^{6}$	$(3.18\pm0.26)\times10^{-2}$	6.9±1.2	94.22±0.26	49.90±0.51	98.78±0.66	101.4±1.0	57.3±0.6
	H3	$(4.91\pm0.18)\times10^{4}$	$(4.83\pm0.13)\times10^{-3}$	98±6.2	89.81±0.51	61.31±1.38	119.85±1.65	125.9±2.6	71.2±1.5
	L13	$(8.30\pm0.11)\times10^{4}$	$(1.00\pm0.014)\times10^{-2}$	121±3.3	90.67±0.53	119.56±3.26	265.96±4.51	276.7±6.4	156.4±3.6
	H25	$(6.89\pm0.16)\times10^{4}$	$(3.11\pm0.038)\times10^{-2}$	450±16	90.76±0.43	277.45±5.21	513.42±6.85	538±10.2	304.1±5.8
MMP-14	2B4	$(1.24\pm0.19)\times10^{4}$	$(7.88\pm0.56)\times10^{-4}$	66±15	85.35±0.62	49.65±1.46	97.51±1.96	105.0±3.1	94.5±2.7
	2B12	$(7.03\pm0.18)\times10^{4}$	$(5.27\pm0.25)\times10^{-3}$	76±5.6	93.79±0.49	57.50±1.13	135.94±1.60	139.3±2.3	125.3±2.1
	1A5	$(4.35\pm0.087)\times10^{4}$	$(2.47\pm0.13)\times10^{-3}$	57±4.1	91.70±0.37	83.49±1.19	169.35±1.70	175.9±2.5	158.3±2.3
	2B10	$(4.01\pm0.29)\times10^{4}$	$(1.85\pm0.097)\times10^{-3}$	46±5.8	88.32±0.38	107.69±2.14	236.76±2.74	249.2±4.1	224.3±3.6
	2D9	$(1.51\pm0.30)\times10^{4}$	$(3.87\pm0.16)\times10^{-3}$	256±62	93.06±0.31	103.57±1.50	258.04±1.88	264.8±2.7	238.3±2.5
	2A6	$(2.36\pm0.21)\times10^{4}$	$(3.02\pm0.11)\times10^{-3}$	128±16	92.22±0.47	199.55±3.73	477.07±4.92	491.7±7.4	442.6±6.7
BACE-1	B3B12	$(6.71\pm0.60)\times10^{5}$	$(6.83\pm0.37)\times10^{-3}$	10±1.5	93.73±0.40	12.70±0.23	31.47±0.29	32.2±0.4	25.6±0.3
	B1A4	$(4.20\pm0.34)\times10^{5}$	$(8.55\pm0.71)\times10^{-3}$	20±3.3	90.69±0.63	25.59±0.82	49.69±0.99	52.0±1.5	41.4±1.2
	B2B5	$(1.80\pm0.23)\times10^{5}$	$(2.42\pm0.27)\times10^{-2}$	134±32	98.08±0.30	32.08±0.40	99.64±0.55	100.2±0.8	79.8±0.6
	B2B2	$(1.77\pm0.18)\times10^{5}$	$(9.10\pm0.19)\times10^{-3}$	52±6.4	95.15±0.13	28.64±0.20	104.88±0.27	106.1±0.4	84.5±0.3
	B2B3	$(9.31\pm1.70)\times10^{4}$	$(9.70\pm0.23)\times10^{-3}$	104±22	96.44±0.59	73.81±1.63	138.00±2.16	140.4±3.2	111.7±2.5
	B2B9	$(1.80\pm0.35)\times10^{5}$	$(2.00\pm0.16)\times10^{-2}$	111±30	97.90±0.20	48.00±0.41	150.86±0.60	151.8±0.8	120.8±0.6
	B2B6	$(1.83\pm0.16)\times10^{5}$	$(5.12\pm0.13)\times10^{-3}$	28±3.1	100.16±0.10	58.19±0.22	157.75±0.33	157.7±0.4	125.5±0.4
	B1B3	$(4.16\pm0.88)\times10^{5}$	$(1.30\pm0.03)\times10^{-2}$	31±7.5	90.10±0.44	115.23±2.04	258.39±2.79	269.4±4.2	214.4±3.4
Alp2	A4A1	$(8.21\pm1.24)\times10^{4}$	$(8.66\pm0.62)\times10^{-4}$	10.5±2.3	93.32±0.53	6.13±0.17	15.44±0.25	15.8±0.3	14.0±0.3
	A4A2	$(8.21\pm0.42)\times10^{3}$	$(8.66\pm0.61)\times10^{-4}$	106±13	94.09±0.34	110.24±1.69	245.31±2.03	251.3±3.0	222.1±2.6
	A4A7	$(5.18\pm0.64)\times10^{3}$	$(2.19\pm0.11)\times10^{-3}$	420±74	85.10±0.52	140.39±3.20	319.04±4.12	340.6±6.7	301.0±5.9
cathepsin B	CBA3	$(4.82\pm0.26)\times10^{6}$	$(7.52\pm0.21)\times10^{-2}$	16±1.3	96.99±0.28	52.77±0.54	129.15±0.73	130.6±1.1	91.0±0.7
	CBA2	$(3.03\pm0.13)\times10^{5}$	$(5.90\pm0.13)\times10^{-2}$	195±13	81.71±0.37	67.08±1.50	162.59±1.74	175.9±2.8	122.6±2.0
	CBA1	$(2.10\pm0.13)\times10^{5}$	$(6.23\pm0.27)\times10^{-2}$	296±31	91.31±0.56	138.10±3.38	284.16±4.04	295.6±6.2	206.0±4.3

Note:

* Association (k_{on}) and dissociation (k_{off}) rates were measured at three Fab concentrations using biolayer interferometry. Averages of k_{on} and k_{off} and their standard errors (S.E.) were calculated with BLItz Pro software for determining dissociation constants K_D ± S.E. Obtained k_{on}, k_{off}, and K_D values are reported as two significant digits in **Table 1** and **Fig S4**.

† The correlations between inhibition percentage and Fab concentration were measured with 1 μM FRET peptide substrates. Using Sigma Plot, data fitting with the 3-parameter sigmoidal equation $y = a / (1+e^{-([I]-I_0)/b})$ generated a, b, I_0 parameters and their S.E. IC_{50} ± S.E. were then calculated for determination of inhibition constants (K_I ± S.E.) using equation $K_I = IC_{50}/(1+[S]/K_m)$, where [S] = 1μM and K_m is the Michaelis-Menten constant of tested protease (**Fig S1**). IC_{50} and K_I values are reported as two significant digits in **Table 1** and **Fig S5**.

Table S3. Yields of representative anti-BACE1 mAbs

mAb		Potency (nM)	Yield (mg/L)
Fab	B3B12	26	0.08
	B1A4	41	0.10
	B2B5	80	0.56
	B2B2	85	1.3
IgG	B3B12	16	2.0
	B1A4	37	2.0

Figure S1:

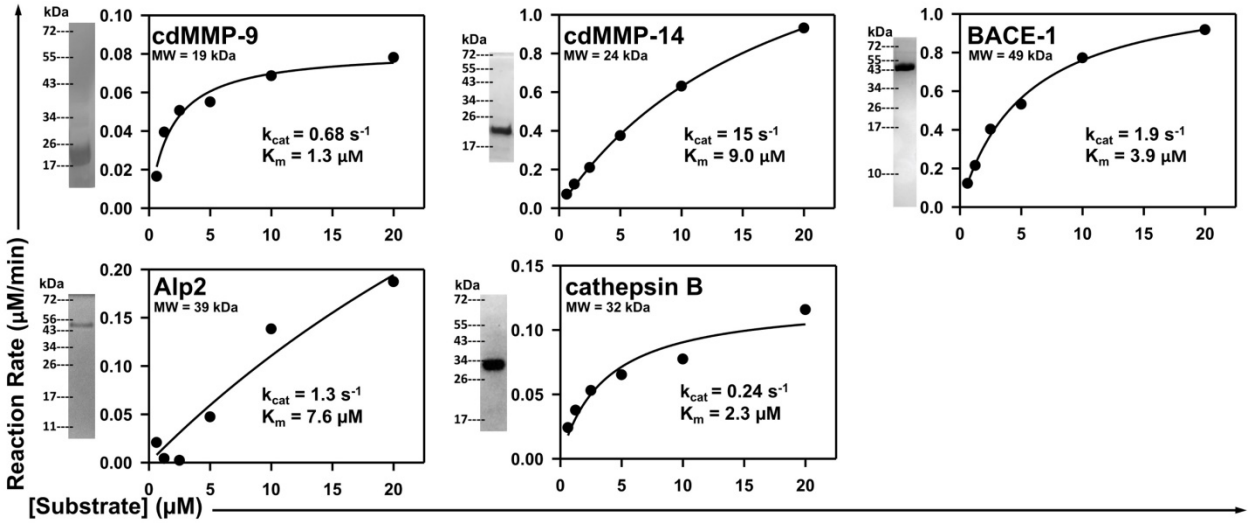

Fig. S1. Periplasmic production of extracellular / catalytic domains of human / fungal proteases in their active soluble format. Purified protases were analyzed by SDS-PAGE, and their enzymatic kinetics were measured with associated FRET peptide substrates.

Figure S2:

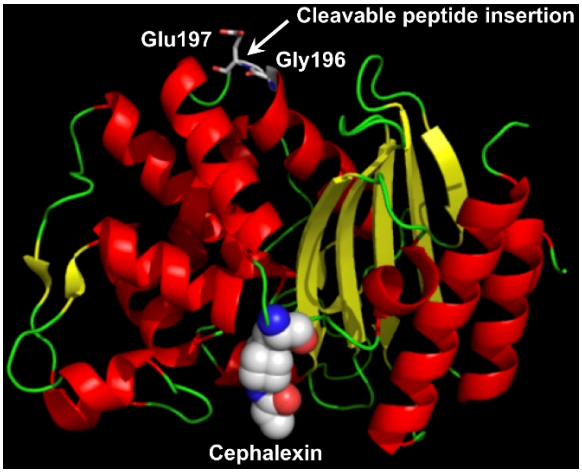

Fig. S2. Design of β-lactamase TEM1 sensor for protease inhibition. Structure (PBD, 4ZJ3) showing that the location of cleavage peptide insertion (between Gly196 and Glu197) is on a loop far away from the active site where inhibitors, e.g. cephalexin, bind. Images were generated by using PyMol.

Figure S3:

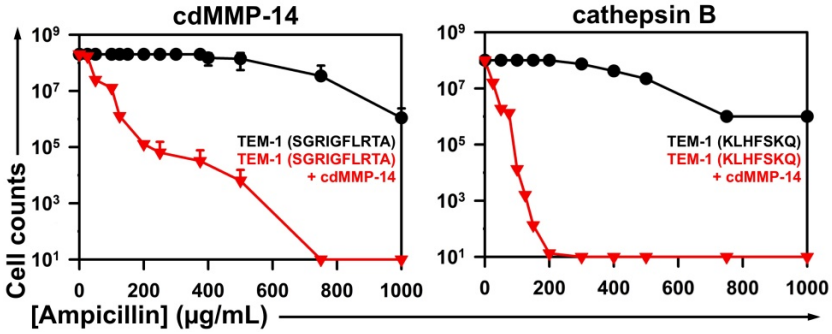

Fig. S3. Selection windows for cdMMP-14 and cathepsin B inhibitors. β-lactamase TEM-1 was modified by insertion of the protease specific cleavage peptide sequences (shown in parentheses) between Gly196 and Glu197 of TEM-1. At 0-1000 μg/mL ampicillin, survival curves of *E. coli* cells transformed with modified TEM-1s without protease genes were measured (black circles), and compared to those for cells co-expressing both modified TEM-1s and the associated proteases (red triangles). Experiments were repeated three times with 2×YT agar plates containing either 2% glucose for cdMMP-14 or 0.1 mM IPTG for cathepsin B.

Figure S4:

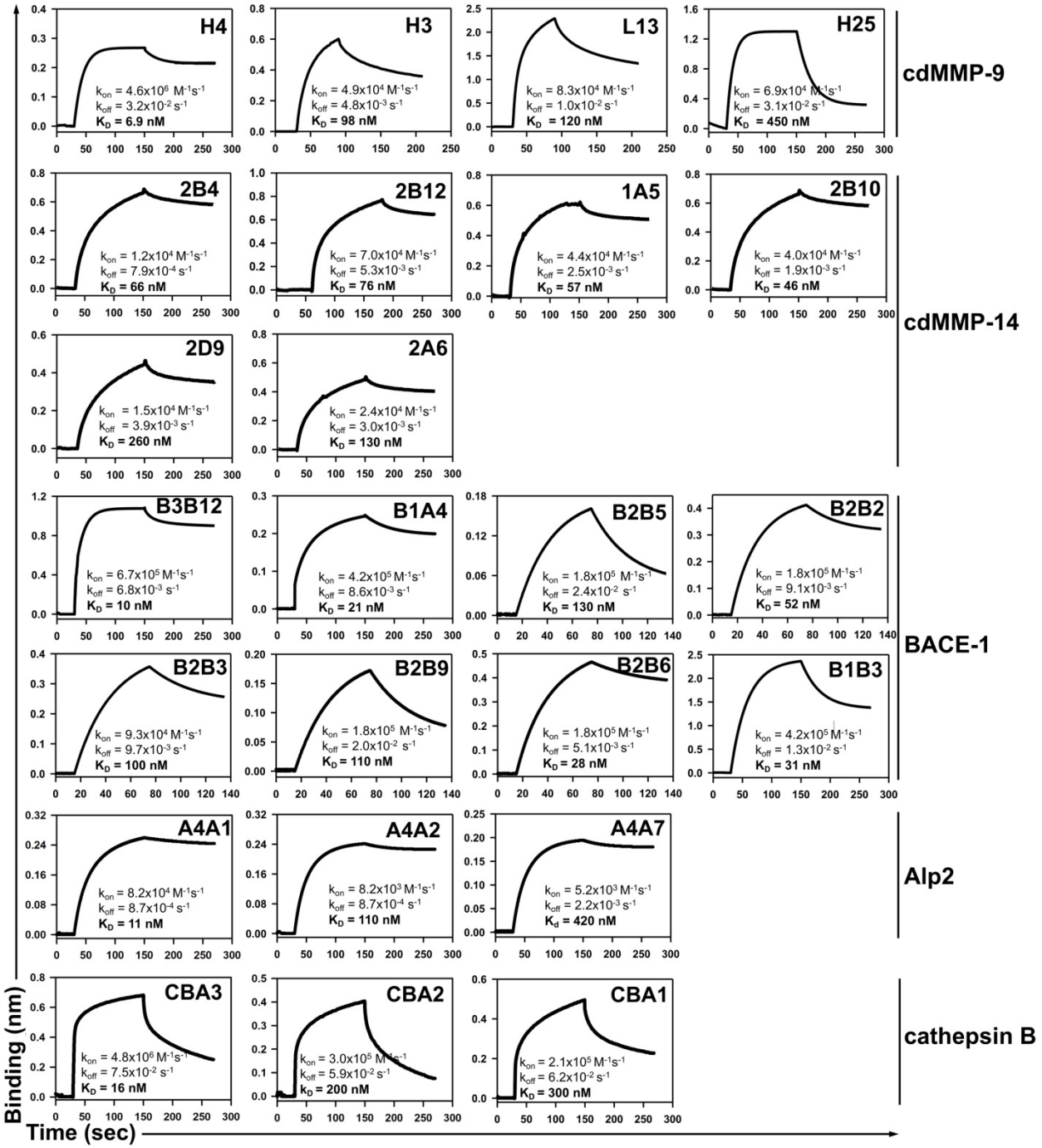

Fig. S4. **Binding kinetics of isolated Fabs to protease targets.** k_{on} and k_{off} values were measured by bio-layer interferometry and used for K_D calculation. Only Fab clones with K_I < 500 nM are shown in the same orders as in **Table 1**. See **Table S2** for statistical analysis.

Figure S5:

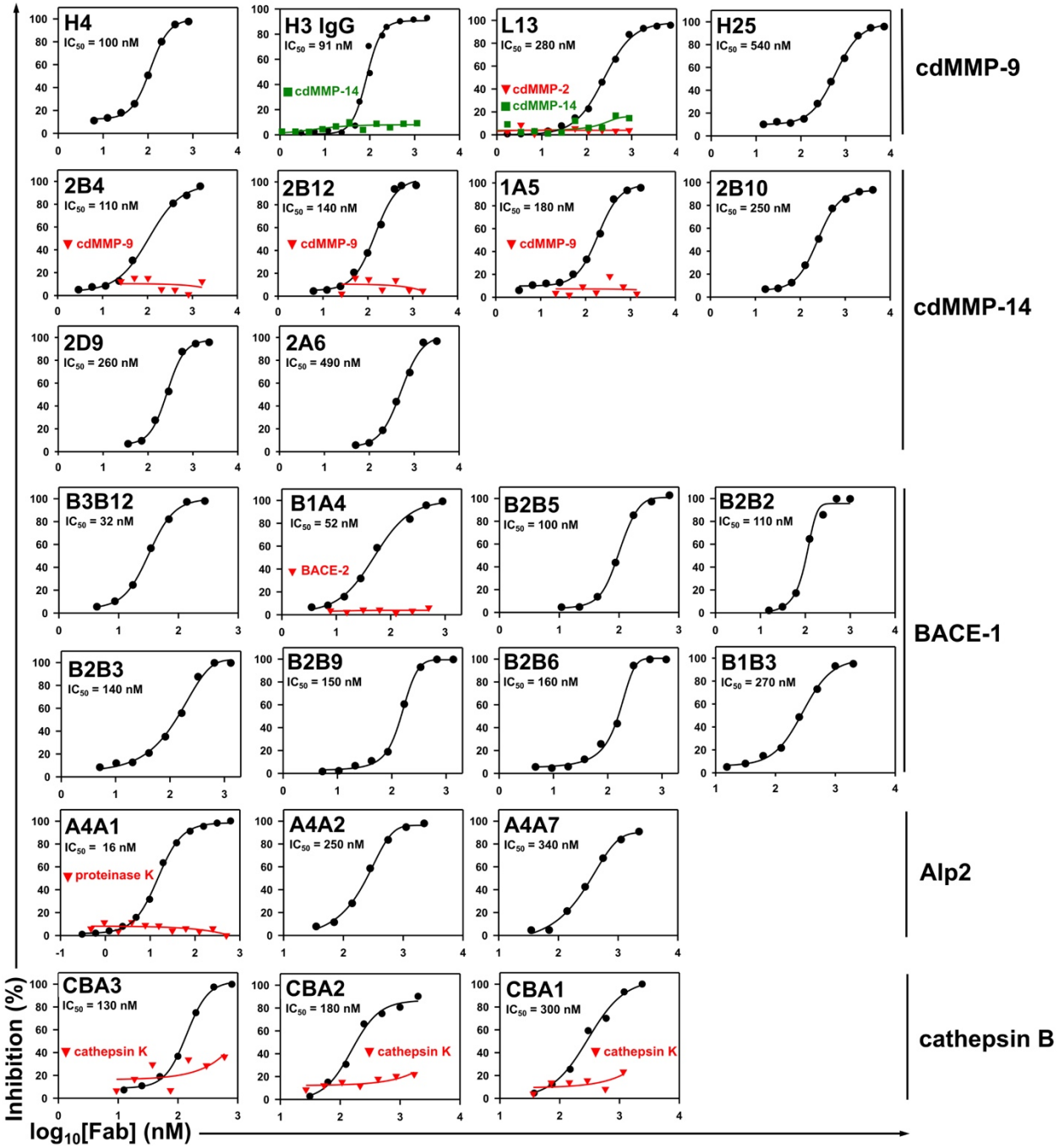

Fig. S5. Inhibition potencies of isolated Fabs. Inhibition IC_{50}s were measured by using FRET peptide substrates. Only Fab clones with K_I < 500 nM are shown in the same orders as in **Table 1**. Inhibition selectivity of representative Fabs was also tested. For anti-cdMMP9 clone H3, its IgG instead of Fab is shown. See **Table S2** for statistical analysis.

Figure S6:

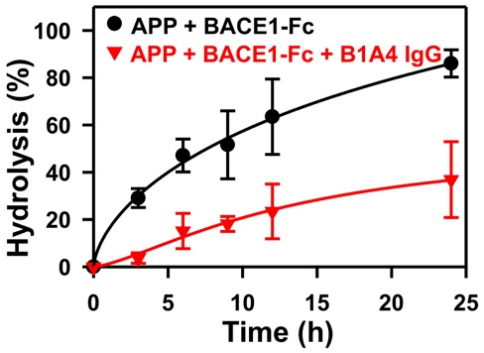

Fig. S6. Inhibitory functions of anti-BACE1 IgG B1A4 on proteolysis of amyloid precursor protein (APP). 5 μM MBP-APP was incubated with 1 μM BACE1-Fc in the absence or presence of 1 μM IgG B1B4 at 37 ^{o}C for 24 h. Samples were taken and analyzed by SDS-PAGE to quantify amounts of remaining APP. Purified BACE1-Fc exhibited catalytic kinetics k_{cat} of 0.23/min and K_m of 1.24 μM.

Figure S7:

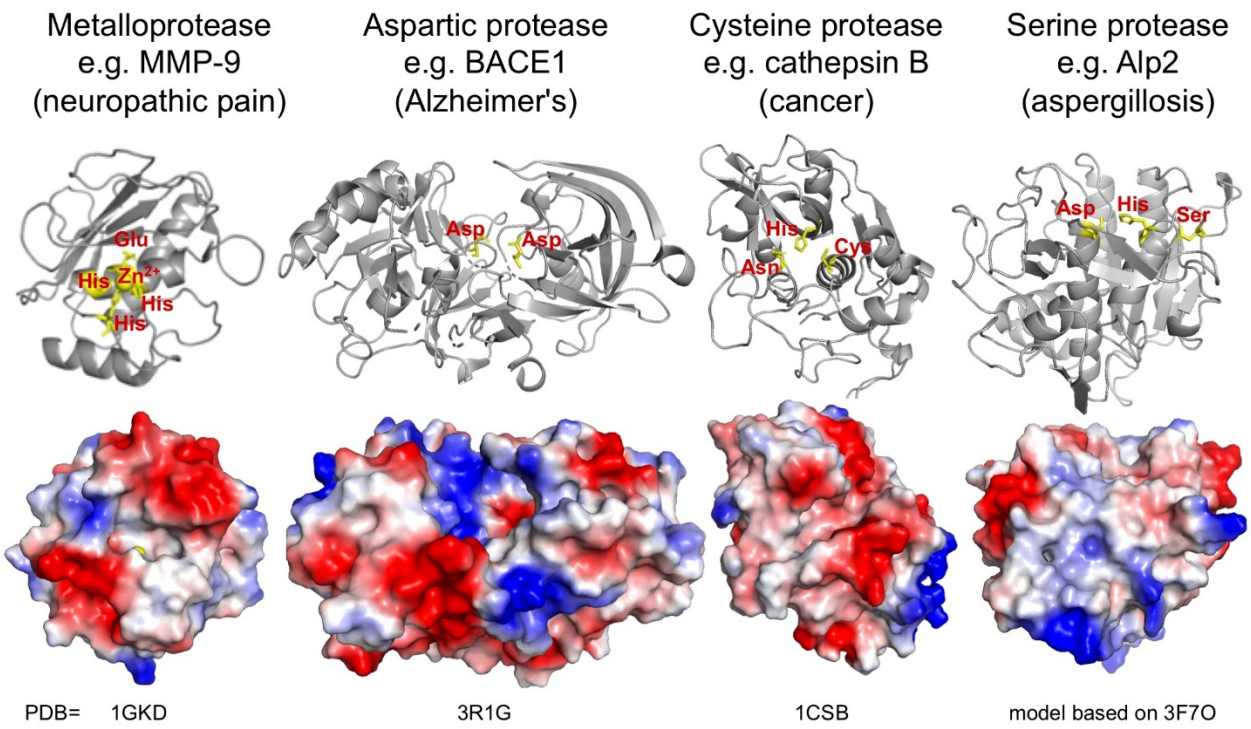

Fig. S7. Active sites and electrostatic surface potentials of representative proteases. Side chains of the catalytic residues are highlighted in yellow. Surface topologies display the reactive clefts or cavities of diverse conformation. Images were generated with indicated PDB files by using PyMol.

Figure S8:

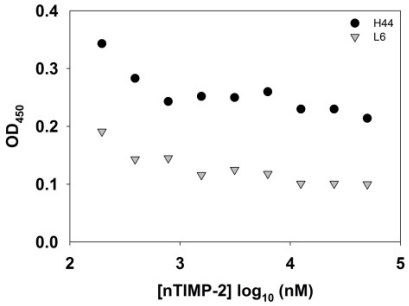

Fig. S8. Competitive ELISA of anti-MMP9 Fabs H44 and L6. 500 nM Fabs were incubated with 200 nM -50 μM nTIMP-2 for 15 min then added to wells coated with cdMMP-9. Captured Fabs were detected with anti-Fab-HRP.