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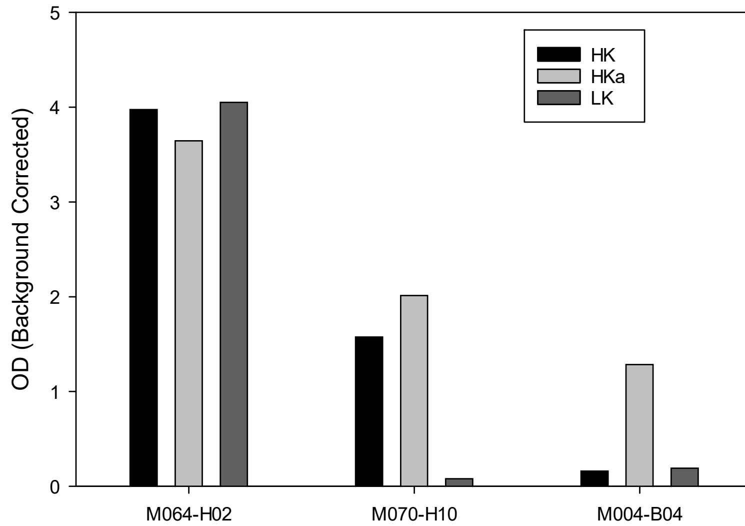
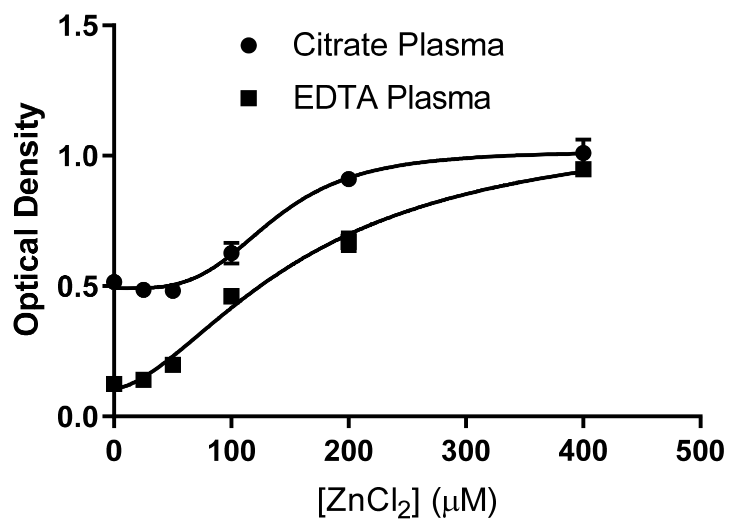
Supplementary Material

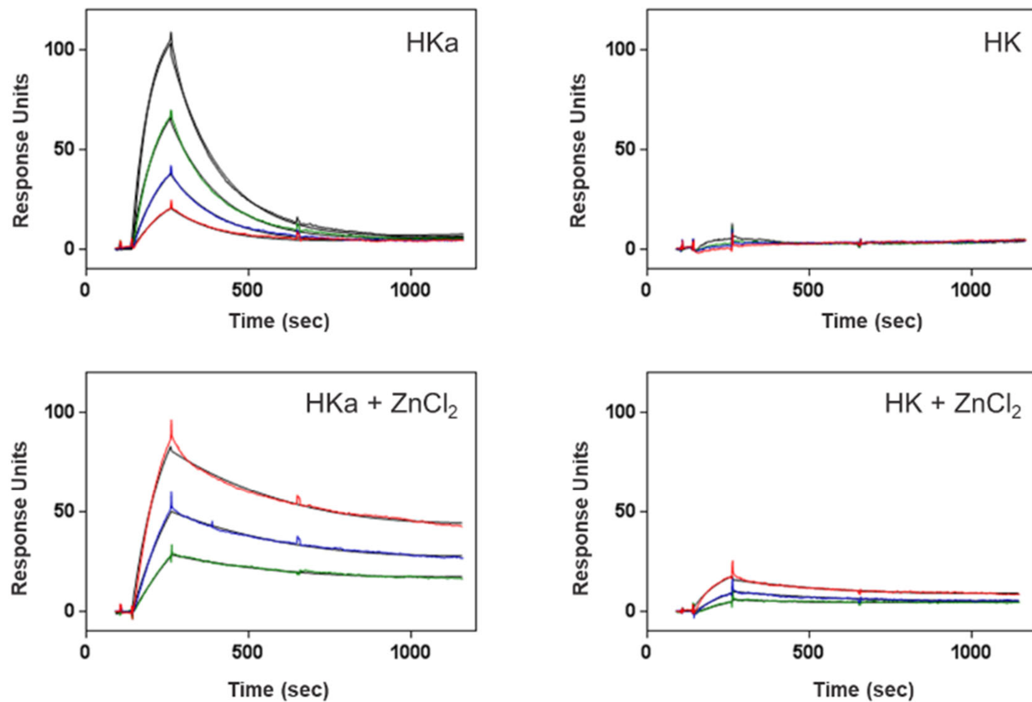
Supplementary Figures



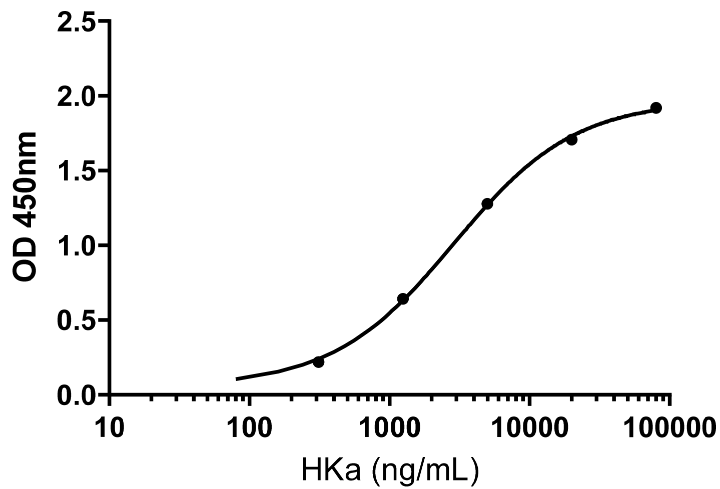
Supplementary Figure 1. Comparison of M4-B4 (aka M004-B04) specificity to 2 other antibodies identified by phage display. HK, high-molecular-weight kininogen; HKa, cleaved high-molecular-weight kininogen; LK, low-molecular-weight kininogen; OD, optical density.



Supplementary Figure 2. The addition of zinc chloride ($ZnCl_2$) increases M4-B4 ELISA signal in ellagic acid treated citrated or EDTA human plasma.

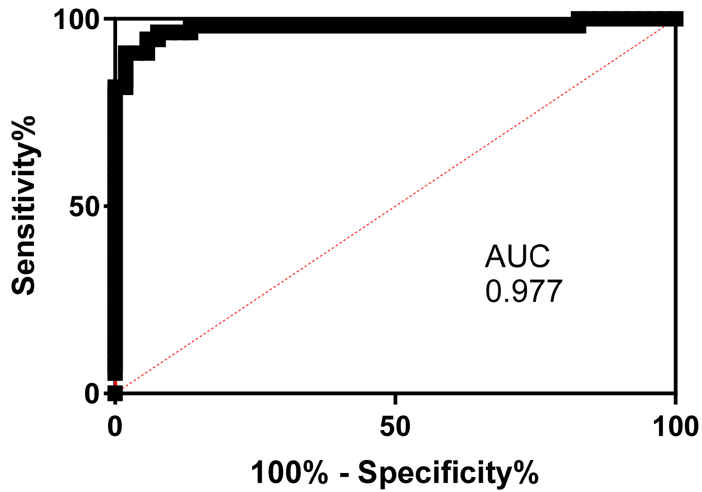


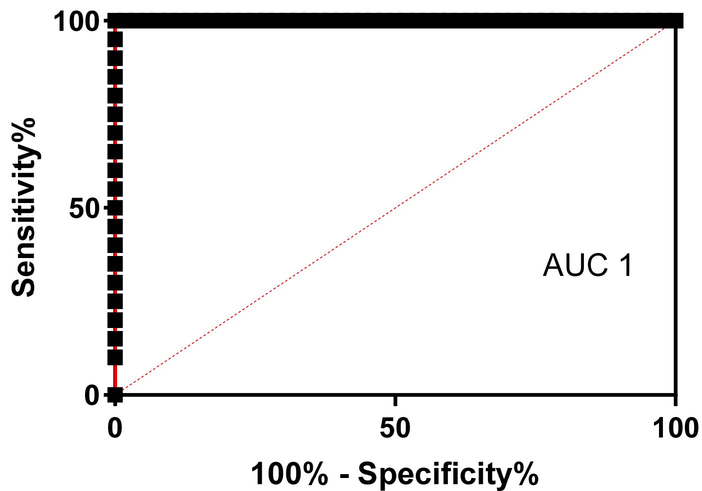
Supplementary Figure 3. Surface plasmon resonance analysis of HKa or HK binding to M4-B4 in the presence or absence of zinc chloride ($ZnCl_2$). Measurement of binding kinetics of M4-B4 to different forms of kininogen were performed using a Biacore 3000 instrument (GE Healthcare Life Sciences) with the detection temperature at 25°C and HBS-P running buffer (10 mM HEPES, pH 7.4, 150 mM sodium chloride, and 0.005% surfactant P20) with and without 1M EDTA or 200 µM $ZnCl_2$. The different forms of kininogen (intact HK and HKa) were immobilized by amine-coupling on a CM5 sensor chip at ~400 response units, and M4-B4 injected for 2 minutes at 50 µL/min followed by a 15-minute dissociation phase. Surfaces for experiments with included $ZnCl_2$ were regenerated with a 32-second pulse of 10 mM glycine (pH 1.5) at 75 µL/min. Kinetic association (k_{on}) and dissociation (k_{off}) constants were obtained by using the Biaevaluation software with the model for the formation of a complex with a 1:1 stoichiometry. HK, high-molecular-weight kininogen; HKa, cleaved high-molecular-weight kininogen.

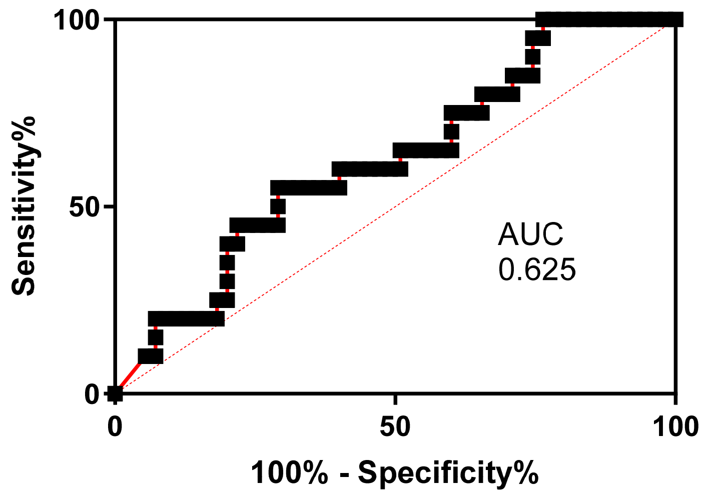


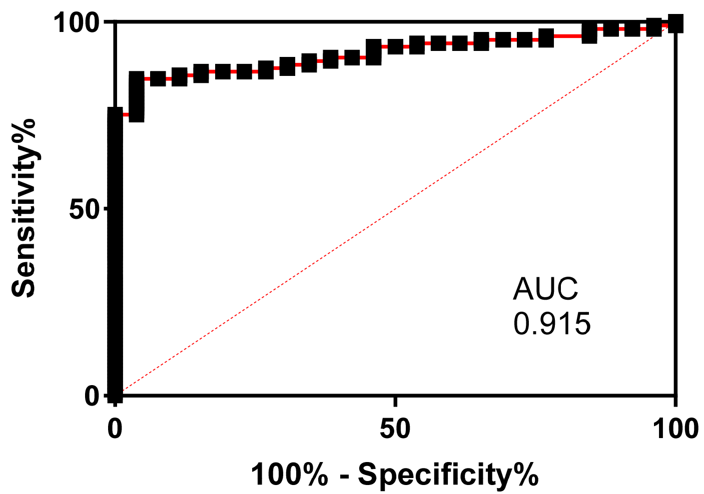
Supplementary Figure 4. Cleaved high-molecular-weight kininogen (HKa) ELISA standard curve. OD, optical density. The standard curve was fit by nonlinear regression analysis to the four parameter logistic equation: $y = d + (a - d)/(1 + \left(\frac{x}{c}\right)^{b})$ where y = OD, a = the minimum value that can be obtained, d = the maximum value that can be obtained, c = the point of inflection, and b = Hill's slope of the curve.

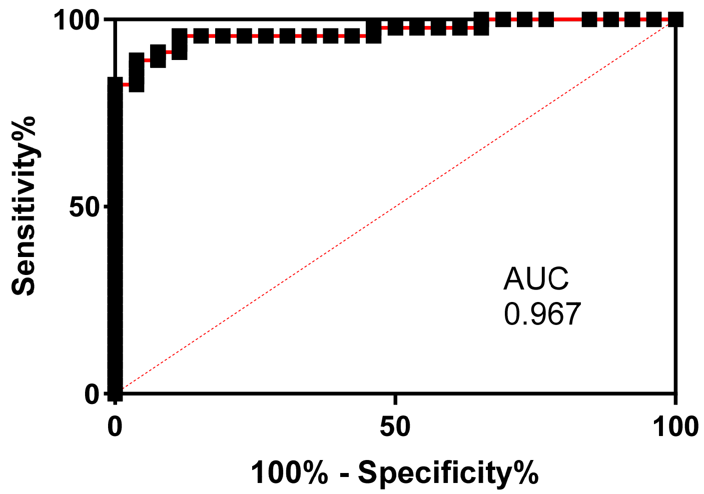
Supplementary Figure 5. ROC curves for plasma HKa in HVs and HAE-C1INH patients. The area under the curve (AUC or C-statistic) is indicated on each curve.

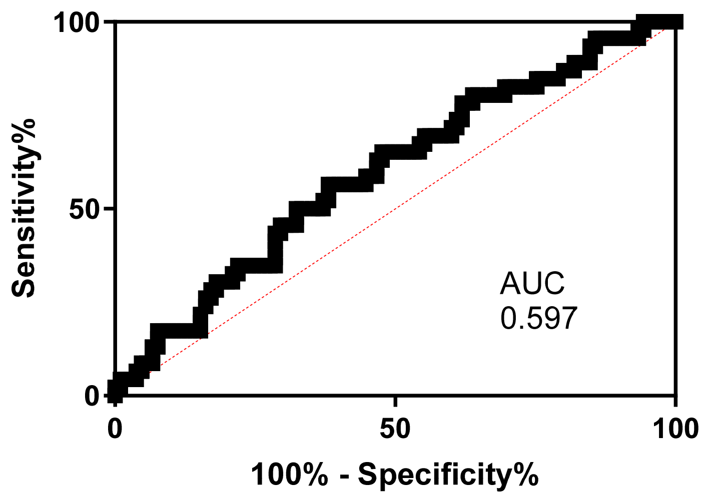





C: HAE-C1INH Basal vs Attack
(Western blot analysis
with sodium citrate plasma)
100
50
0
Sensitivity%
0
50
100
100% - Specificity%
AUC
0.625


D: HV vs HAE-C1-INH Basal
(Western blot analysis
with SCAT169 plasma)
100
50
0
Sensitivity%
0
50
100
100% - Specificity%
AUC
0.915


E: HV vs HAE-C1INH Attack
(Western blot analysis
with SCAT169 plasma)
100
50
0
Sensitivity%
AUC
0.967
0
50
100
100% - Specificity%


F: HAE-C1INH Basal vs Attack
(Western blot analysis
with SCAT169 plasma)
100
50
0
Sensitivity%
AUC
0.597
0
50
100
100% - Specificity%

G: HV vs HAE-C1-INH Basal
(ELISA with sodium citrate plasma)

Sensitivity%

100
50
0

AUC
0.795

0 50 100

100% - Specificity%

H:HV vs HAE-C1INH Attack
(ELISA with sodium citrate plasma)

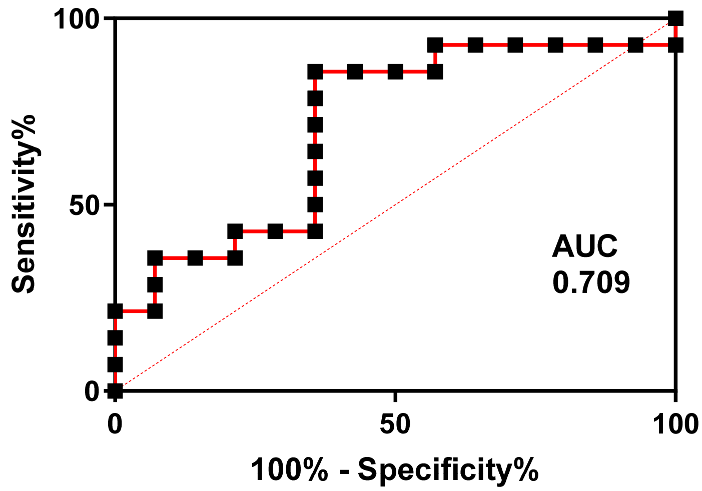
Sensitivity%

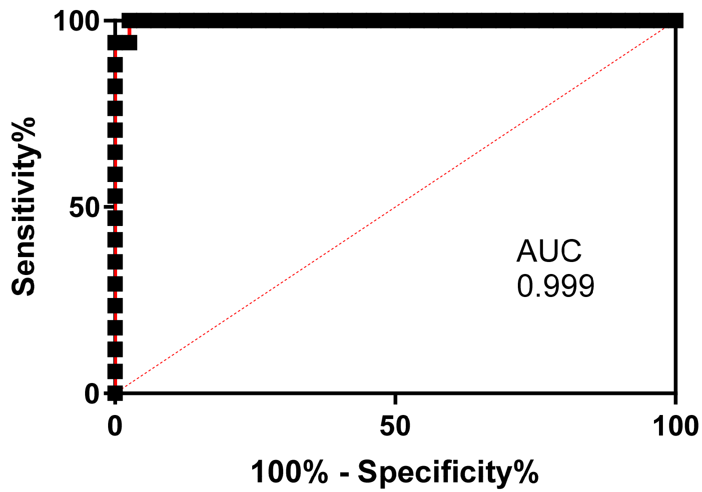
100
50
0

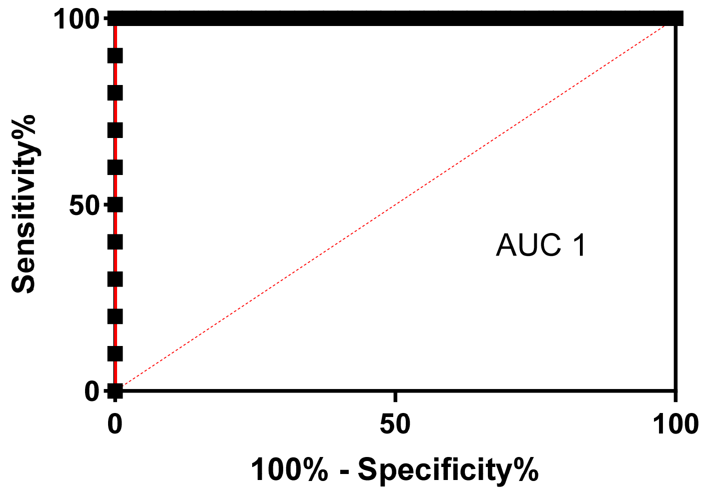
AUC
0.866

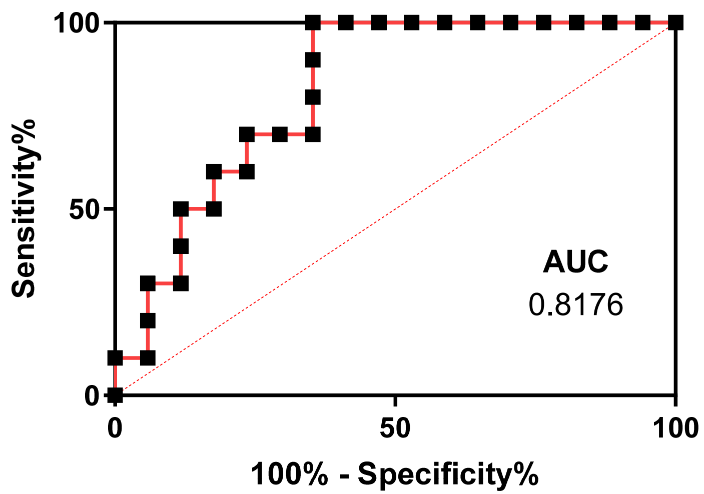
0 50 100

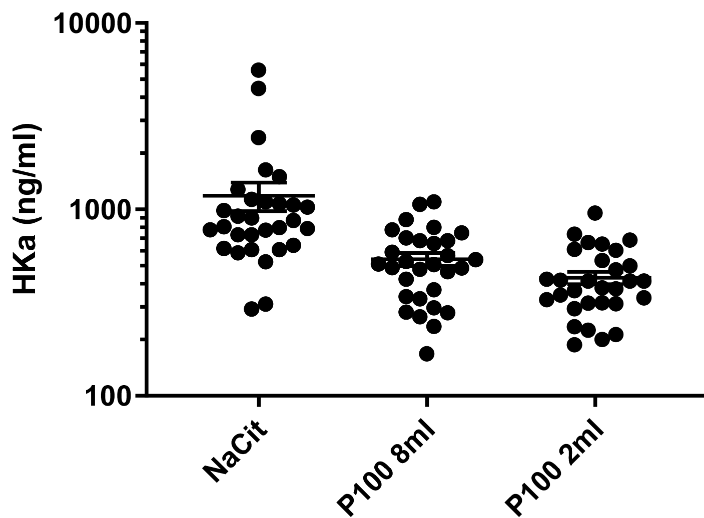
100% - Specificity%


I: HAE-C1INH Basal vs Attack
(ELISA with sodium citrate plasma)
100
50
0
0
50
100
Sensitivity%
100% - Specificity%
AUC
0.709

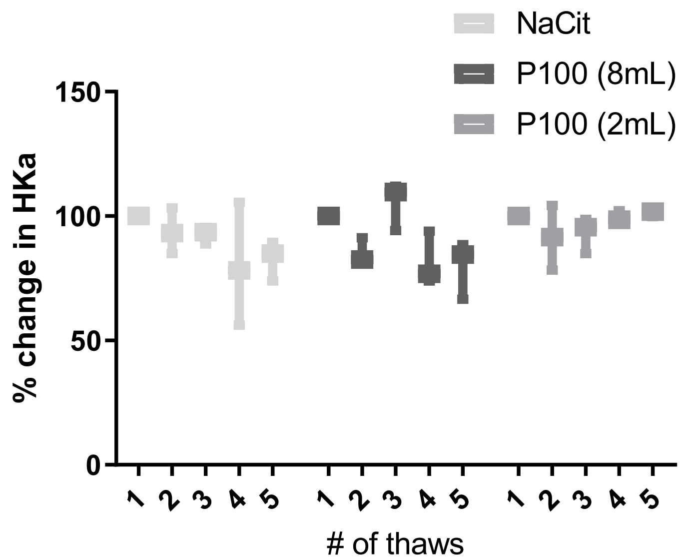

J: HV vs HAE-C1-INH Basal
(ELISA with SCAT169 Plasma)
100
50
0
0
50
100
Sensitivity%
100% - Specificity%
AUC
0.999


K: HV vs HAE-C1INH Attack
(ELISA with SCAT169 Plasma)
100
50
0
Sensitivity%
AUC 1
0
50
100
100% - Specificity%


L: HAE-C1INH Basal vs Attack
(ELISA with SCAT169 Plasma)
100
50
0
Sensitivity%
AUC
0.8176
0
50
100
100% - Specificity%



Supplementary Figure 6. Comparison of cleaved high-molecular-weight kininogen (HKa) levels in healthy volunteers by M4-B4 enzyme-linked immunosorbent assay (ELISA) in citrated plasma versus plasma collected in P100 tubes (either 8 mL or 2 mL total volumes from BD Biosciences) from 30 individual healthy volunteers. Coat Nunc MaxiSorp plates with 3 µg/mL of M4-B4 capture antibody in 0.2 M carbonate bicarbonate and let incubate at 4°C, shaking, overnight. On the day of the assay, wash coated plates with a solution of 1× PBS and 0.05% Tween 20 at least 3 times. Blot plates to remove as much of the washing buffer as possible and block plates with 300 µL of 1× PBS, 5% BSA and 0.05% Blocking Reagent solution and let it incubate for 1 hour, shaking at RT. During the incubation period, samples, standard curves and controls can be prepared. Dilute reference standard, HKa in sample dilution buffer (1× PBS, 1% BSA, 0.01% Blocking Reagent) at a starting concentration of 250 ng/mL, proceeding with 10 more 1:2 serial dilutions and ending the standard curve with a dilution buffer blank. All plasma samples were diluted and 1:300, 1:600, and 1:1200. After an hour of blocking, plates can be washed again using the process described above and 100 µL of samples, standards and controls can be added to the plate. Immediately after an hour of sample incubation, plates are washed and 0.5 µg/mL of 11H05 detection antibody is added to each well and incubated for 1 hour, shaking at RT. In order to detect a signal, a 1:100,000 solution of Goat anti-mouse IgG-HRP secondary antibody needs to be added after the detection step and incubated for another hour. Once the ELISA is complete, the plate is washed and 50 µL of TMB Substrate solution is added to each well. After 10–15 minutes of color development, 100 µL of Sulfuric Acid 2N is added to each well to stop the reaction. The absorbance is read immediately in a SpectraMax microplate reader at 450 nm with a 630-nm correction. HRP, horseradish peroxidase.

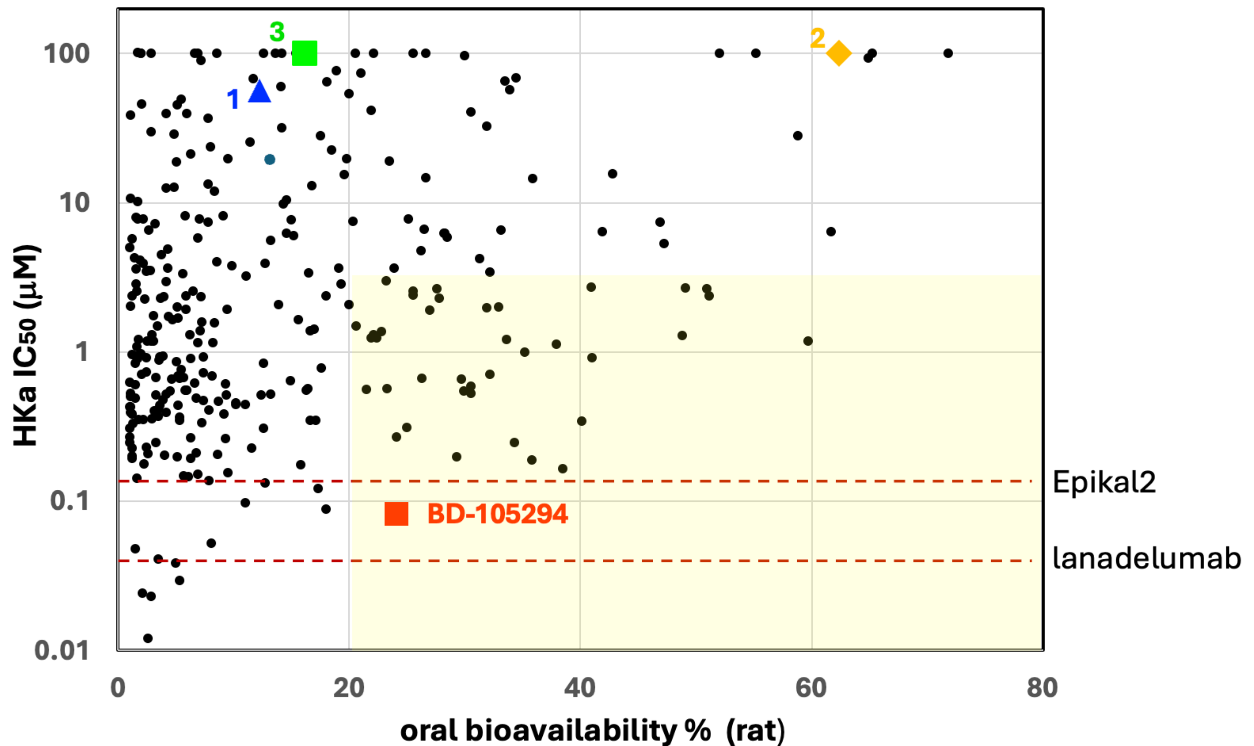


Supplementary Figure 7. Comparison of HKa levels in plasma after multiple freeze thaws by M4-B4 ELISA in citrated plasma versus plasma collected in P100 tubes (either 8 mL or 2 mL total volumes from BD Biosciences) from 30 individual healthy volunteers. NaCit, sodium citrate; HKa, cleaved high-molecular-weight kininogen.

559B-M0004-B04 HC IgG
EVQLLESGGGLVQPGGSLRLSCAASGFTFSFYVMVWVRQAPGKGLEWVSGISPSGGNT
AYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARKLFYYDDTKGYFDFWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPC
PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ
VYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL
YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

559B-M0004-B04 LC (Lambda)MW
QYELTQPPSASGTPGQRVTLSCSGSSSNIGSNYVYWYQQLPGTAPKLLIYRNNQRPSGVP
DRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLNGRVFGGGTKLTVLGQPKAAPS
VTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYA
ASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS

Supplementary Figure 8. M4-B4 amino acid sequence.



Supplementary Figure 9. Comparison of PKa inhibitors potencies versus oral bioavailability in rat. Pharmacokinetic assessment of small-molecule plasma kallikrein inhibitors were performed with Sprague Dawley male rats. Pharmacokinetic studies were performed by intravenous (IV) injection and oral (PO) gavage. Three animals were included in each dose group for PO and IV studies. Blood sampling was performed at 9 times points for IV and 8 times point for PO administration. Previously reported PKa inhibitors 1, 2, and 3 in Supplementary Table 4 are highlighted along with a previously unreported PKa small molecule inhibitor BD-105294. The IC50 of EPI-KAL2 and lanadelumab are indicated by the red dashed lines and as both are biologic PKa inhibitors were not tested for oral bioavailability.

Supplementary Tables
Supplementary Table 1.

Plasma HKa levels in HVs and patients with HAE-C1INH	**HKa Western blot**					
	Citrated plasma (Figure 4A)			SCAT169 plasma (Figure 4B)		
	HV	Basal	Attack	HV	Basal	Attack
N	52	55	20	26	105	46
Minimum (%HKa)	2.9	6.2	31.91	3.2	2.6	5.8
Maximum (%HKa)	29.5	100	100	12.7	81	100
Median (%HKa)	9.4	49.5	62.3	6.6	19.9	24.2
Mean (%HKa)	10.2	51.4	61.5	6.6	21.6	26.4
SD (%HKa)	4.9	23	23.8	2	13	16.8
	HKa ELISA					
	[HKa] in citrated plasma (Figure 4C)			[HKa] in SCAT169 plasma (Figure 4D)		
	HV	Basal	Attack	HV	Basal	Attack
N	24	14	14	39	17	10
Minimum (ng/mL)	325.9	1831	1631	215.1	523.7	1645
Maximum (ng/mL)	18641	13057	30776	540.4	5189	8758
Median (ng/mL)	1111	3833	5304	357.4	1299	3167
Mean (ng/mL)	2966	4994	9389	370.7	1780	3599
Mean (%HKa)[a]	3.6	6.1	11.5	0.5	2.2	4.4
Mean (nM)[b]	27.0	45.4	85.4	3.4	16.2	32.7
SD (ng/mL)	4263	3288	8023	91.83	1246	2219

[a] Expressed as a percent of total HK 82,000 ng/mL [Scott CF, Shull B, Muller-Esterl W, Colman RW. Rapid direct determination of low and high-molecular-weight kininogen in human plasma by particle concentration fluorescence immunoassay (PCFIA). *Thromb Haemost.* 1997;77:109-118.]

[b] Calculated using a HK molecular weight of 110 kDa.

HAE-C1INH, hereditary angioedema due to a deficiency in total (type I) or functional C1 inhibitor protein (type II); HK, high-molecular-weight kininogen; HKa, cleaved high-molecular-weight kininogen; HV, healthy volunteer; SD, standard deviation.

Supplementary Table 2. Kinetic constants obtained from surface plasmon resonance analysis of HKa or HK binding to M4-B4 in the presence or absence of $ZnCl_2$

		k_a (1/Ms)	**k_d (1/s)**	**K_D (M)**	**Chi_2**
No added $ZnCl_2$	Replicate 1	1.14E+06	6.70E-03	5.90E-09	0.254
	Replicate 2	9.84E+05	6.16E-03	6.26E-09	0.828
	Average	1.06E+06	6.43E-03	6.08E-09	0.541
	SD	1.10E+05	3.82E-04	2.55E-10	
With 200 μM $ZnCl_2$	Replicate 1	1.15E+06	1.25E-03	1.09E-09	1.56
	Replicate 2	1.38E+06	1.72E-03	1.24E-09	0.852
	Replicate 3	1.77E+06	1.56E-03	8.81E-10	0.27
	Average	1.58E+06	1.64E-03	1.06E-09	0.894
	SD	2.76E+05	1.13E-04	2.54E-10	

HK, high-molecular-weight kininogen; HKa, cleaved high-molecular-weight kininogen; SD, standard deviation; $ZnCl_2$, zinc chloride.

Supplementary Table 3. Comparison between plasma HKa levels between HVs and HAE-C1INH patients

		HV vs basal	HV vs attack	Basal vs attack
%HKa in citrated plasma by Western blot	Mann-Whitney test	$P<0.0001$	$P<0.0001$	$P=0.1022$
	ROC C-statistic	0.9773	1.00	0.6245
	Difference between mean (%HKa)	41.2	51.3	10.1
%HKa in SCAT169 plasma by Western blot	Mann-Whitney test	$P<0.0001$	$P<0.0001$	$P=0.0579$
	ROC C-statistic	0.9147	0.9666	0.5971
	Difference between mean (%HKa)	15	19.8	4.8
HKa in citrated plasma by ELISA	Mann-Whitney test	$P=0.0021$	$P<0.0001$	$P=0.0062$
	ROC C-statistic	0.7946	0.8661	0.7092
	Difference between mean (ng/mL)	2232	4052	1471
HKa in SCAT169 plasma by ELISA	Mann-Whitney test	$P<0.0001$	$P<0.0001$	$P=0.0056$
	ROC C-statistic	0.9985	1.00	0.8176
	Difference between means (ng/mL)	941.6	2810	1868

HAE-C1INH, hereditary angioedema due to a deficiency in total (type I) or functional C1 inhibitor protein (type II); HKa, cleaved high-molecular-weight kininogen; HV, healthy volunteer; ROC, receiver operator characteristic.

Supplementary Table 4. Comparison of PKa inhibitor potency

PKa inhibitor	Ki (nM) or IC_{50} (nM) [a]	HKa ELISA IC_{50} (nM)[h]	Fluorogenic peptide IC_{50} (nM)[i]
Lanadelumab	0.12 [b]	40	22
EPI-KAL2 (ecallantide surrogate)	0.1 [c]	150	55
PKa Inhibitor 1	0.9 [d]	60,000	34
PKa Inhibitor 2	6 (IC_{50})[e]	>100,000	124
PKa Inhibitor 3	2.7 (IC_{50})[f]	>100,000	275
BD-105294	0.11 (IC_{50})[g]	82	57

[a]Ki (inhibition constant) or half maximal concentration (IC_{50}) values were collated from literature or internal measurements.
[b]Kenniston JA, et al. *J Biol Chem*. (2014) 289, 23596-608.
[c]Markland W, et al. *Biochemistry.* (1996) 35, 8058-67.
Kotian PL, et al. *J Med Chem*. (2021) 64, 12453-12468.
[e]Davie RL, et al. *J Med Chem*. (2022) 65, 13629-44.
[f]Kalfus I, et al. *J Allergy Clin Immunol.* (2017) 139(2):AB378.
[g]IC_{50} value generated internally using purified enzymes similar to the method reported in Kenniston JA, et al. *J Biol Chem*. (2014) 289(34):23596-608.
[h]IC_{50} values determined using the cleaved high-molecular-weight kininogen (HKa) ELISA described here with FXIIa activation within 90% human plasma.
[i]IC_{50} values determined internally using 90% human plasma activated with FXIIa and monitored plasma kallikrein (PKa) activity using the synthetic fluorescent substrate Pro-Phe-Arg-aminomethylcoumarin (100 μM).

Supplementary Methods

Synthesis of BD-105294: 1-((6-cyclopropylimidazo[1,2-b]pyridazin-2-yl)methyl)-N-(2-fluoro-3-methoxy-6-(1H-tetrazol-1-yl)benzyl)-1H-1,2,3-triazole-4-carboxamide

To a solution of (2-fluoro-3-methoxy-6-(1H-tetrazol-1-yl)phenyl)methanamine[1] (4.4 g, 19.7 mmol) in dry DMF (50 mL) was added 1-((6-cyclopropylimidazo[1,2-b]pyridazin-2-yl)methyl)-1H-1,2,3-triazole-4-carboxylic acid[2] (4.6 g, 16.4 mmol), HOBT (2.9 g, 21.6 mmol), EDCI (4.1 g, 21.6 mmol) and DIPEA (6.3 g, 49.2 mmol). The reaction was stirred at room temperature for 16 h. After the reaction was completed, the solution was poured into water (500 mL) slowly, and the white solid was precipitated. The solid was filtered, the filtered cake was washed with H_2O (300 mL) and dried to give crude, which was triturated with (DCM / MeOH = 20 / 1, 50 mL) to give 1-((6-cyclopropylimidazo[1,2-b]pyridazin-2-yl)methyl)-N-(2-fluoro-3-methoxy-6-(1H-tetrazol-1-yl)benzyl)-1H-1,2,3-triazole-4-carboxamide (4.6 g, yield: 57.5%). ESI-MS [M +H]+: 490.1. Purity: 99.5 %. 1H NMR (400 MHz, DMSO) δ 9.74 (s, 1H), 8.75 (t, J = 5.2 Hz, 1H), 8.50 (s, 1H), 8.18 (s, 1H), 7.92 (d, J = 9.4 Hz, 1H), 7.39 – 7.32 (m, 2H), 7.10 (d, J = 9.5 Hz, 1H), 5.73 (s, 2H), 4.29 (d, J = 5.0 Hz, 2H), 3.92 (s, 3H), 2.20 – 2.14 (m,, 1H), 1.08 – 1.04 (m, 2H), 0.98 – 0.96 (m, 2H).

1. Davie, R.L; Edwards, H.J; Evans, D.M.; Hodgson, S.T.; Pethen, S.J; Rooker, D.P. Enzyme Inhibitors. US202/0275 A1, January 6, 2022.
2. Papaioannou, N.; Fink, S.J.; Miller, T.A.; Shipps, G.W.; Travins, J.M.; Ehmann, D.E.; Rae, A; Ellard, J.M. Inhibitors of Plasma Kallikrein and Uses Thereof. US 10730874 B2, August 4, 2020.