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Kinetic analysis of renin and its inhibitors by detecting double-labelled peptidic substrates with an immunoassay†

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The proteolytic activity of renin is a key element in the regulation of blood pressure and a main target for inhibitor design. Currently, the activity of renin and its inhibitors is mainly analyzed using radioimmunoassays or FRET-substrates, which both have their limitations. Here, a novel kinetic assay is presented that combines the advantages of a homogeneous proteolytic reaction and a robust heterogeneous detection in a sandwich immunoassay format. The proteolysis in solution is not influenced by surface interactions and yields accurate kinetic values, while the specific detection of the cleavage products on a microtiter plate strongly reduces interference by concomitant substances and allows for a self-referenced signal readout. A new enzyme kinetic scheme for the inhibition of renin has been developed and validated by using the model inhibitor pepstatin. This kinetic analysis is amenable to parallelization for large-scale inhibitor screening. Furthermore, it can be easily adapted to inhibitors of other medically important proteases.

Introduction

Renin is a monomeric 37 kDa aspartyl-protease that initiates the renin–angiotensin system (RAS). This protease cascade regulates homeostatic processes, such as blood pressure and fluid volume, but is also involved in the development and progression of fibrotic and hypertrophic diseases.[1] Renin is released from the kidneys into the circulation and specifically cleaves the N-terminal part of the 50 kDa glycoprotein angiotensinogen. The N-terminal decapeptide angiotensin I is further processed by angiotensin-converting enzyme (ACE) to the octapeptide angiotensin II. By binding to the AT_1 receptor, angiotensin II induces vasoconstriction as well as renal sodium and water retention, which increase blood pressure. In particular, the central role in the regulation of blood pressure has urged the development of drugs that inhibit RAS compounds. Renin, the first and rate-limiting step of the RAS, has long been considered as the preferable target for inhibition.[2] Until recently, however, only ACE inhibitors or AT_1 receptor blockers were available for the treatment of hypertension that are implicated in adverse effects.[3] For example, ACE inhibitors also inhibit the proteolysis of bradykinin, which may result in cough and angioedema, and angiotensin II can still be produced by other proteases. The action of angiotensin II, on the other hand, cannot be completely suppressed by AT_1 blockers as it can bind to different receptor subtypes. Consequently, when the first orally available renin inhibitor aliskiren was approved in 2007, it was considered an important hallmark in the more than 100 year-old history of renin and sparked renewed interest in its activity and inhibition.[4]

Haber *et al.*[5] introduced the first reliable radioimmunoassay for measuring the activity of plasma renin in 1969 that – in different variations – is still widely used today.[6] For this competitive immunoassay, the product angiotensin I is captured by an immobilized antibody. Then, the radiolabeled angiotensin I is added, which binds to the remaining free sites of the capture antibody and can be measured with a scintillation counter. To avoid radioactive materials other – mainly homogeneous – assay formats have been developed, *e.g.* based on HPLC-electrospray-tandem mass spectrometry,[7] fluorogenic substrates[8–10] or FRET.[11–13] For fluorogenic substrates, however, a fluorophore must be placed next to the cleavage site, and FRET substrates are only quenched efficiently if the distance between the donor and acceptor is typically in the range of 2 to 6 nm.[14] In particular, peptidic substrates of renin that are derived from the large protein sequence of angiotensinogen require long sequences of at least eight amino acids to show significant hydrolysis rates,[15] and considerable effort has been devoted to identifying good FRET substrates.[13]

We have recently introduced a new stability test for peptides that imposes no restriction on the length of the amino acid sequence and combines the advantages of homogeneous and heterogeneous assay formats.[16] First, double-labelled peptides are subjected to proteolytic degradation in solution (homogeneous

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phase). The peptide is then transferred and immobilized on a microtiter plate *via* its N-terminal label.[17] If the peptide has not been cleaved, the C-terminal label binds to an enzyme-amplified detection system. If the peptide has been cleaved, by contrast, it does not contain the C-terminal label and no signal is observed. The ratio of cleaved to uncleaved peptide defines the stability of the peptide in a biological sample. Here, we employ this detection scheme to accurately determine the proteolytic activity of renin in parallel, and demonstrate how it can be used to elucidate renin–inhibitor interactions with the model inhibitor pepstatin.[18–20]

Experimental

Buffers and reagents

L(ite)-PBS: 10 mM sodium phosphate, pH 7.0, 10 mM NaCl; D(ulbecco's)-PBS: 2.7 mM KCl, 1.5 mM KH_2PO_4, 136 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.3; maleate buffer: 50 mM maleic acid, 33.5 mM Tris, 10 mM EDTA, adjusted to pH 6.0 with NaOH. The monoclonal anti-2,4-D antibody clone E2/G2 was a kind gift from Milan Fránek.[21] Recombinant human renin (50 μg ml^{-1} in 50 mM aqueous 3-(*N*-morpholino)propanesulfonic acid, pH 7.0) was purchased from Anaspec (http://www.anaspec.com); horseradish peroxidase-labelled streptavidin (SA-HRP, 1 mg ml^{-1} in 50 mM $NaHCO_3$, pH 8.3) from Vector Laboratories (http://www.vectorlabs.com); pepstatin from Roche (http://www.roche.de); and 3,3′,5,5′-tetramethylbenzidine (1-Step™ Ultra TMB-ELISA) from Pierce Biotechnology (http://www.piercenet.com).

Peptide synthesis of renin substrates

Peptides were synthesized on a tentagel amide resin (0.24 mmol g^{-1}; Intavis, http://www.intavis.com) at room temperature (rt) using standard fluorenylmethoxycarbonyl (Fmoc) protection chemistry and an automated multiple peptide synthesizer (MultiPep RS, Intavis). First, 1.3 g of the resin was swollen in 50 ml of a solvent mixture (7 : 3, v/v) of dichloromethane and dimethylformamide (DMF). Then, 3 μmol of the resin were transferred to wells of a filter bottom microtiter plate (Intavis, 96 well reaction plate, 2–5 μmol) and washed three times with 300 μl of DMF. The resin was Fmoc-deprotected two times for 5 min with 170 μl of 20% (v/v) piperidine in DMF, and washed ten times with 250 μl of DMF. A solvent mixture of 24 μl of 0.6 M Fmoc-amino acid (Iris Biotech, http://www.iris-biotech.de) in DMF, 8 μl of 4 M 4-methylmorpholine in DMF, and 23 μl of 0.6 M 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (Iiris Biotech) in DMF was applied to the resin two times for 30 min. The resin was washed three times with 300 μl of DMF, incubated for 5 min with 120 μl of 5% (v/v) acetic anhydride in DMF to acetylate unreacted terminal amino groups, and washed six times with 250 μl of DMF. The synthesis cycles were repeated to assemble individual peptides in each well. After a final deprotection step, the resin was washed ten times with 250 μl of DMF. Terminal amino groups of the peptides were acetylated by adding two times 120 μl of 5% acetic anhydride (v/v) in DMF for 5 min. The resin was washed eight times with 250 μl of DMF, and six times with 150 μl of dichloromethane. After extracting for 5 min, the resin was dried *in vacuo* for 12 h. The resin was incubated for 10 min, 30 min and 90 min with 100 μl of a solvent mixture (v/v) of 92.5% trifluoroacetic acid, 5% triisobutylsilane, and 2.5% water to release the peptides into solution, which was then collected in a 96-well plate (MegaBlock, Sarstedt, http://www.sarstedt.com) and dried *in vacuo*. The peptides were incubated with 1250 μl of cold methyl *tert*-butyl ether for 12 h, the supernatants were removed, and the peptides were dried *in vacuo*. A stock solution of 3 mM peptide in DMF was prepared and analyzed by reversed phase HPLC (HP Agilent 1100, http://www.chem.agilent.com)/electrospray ionization mass spectrometry (ThermoQuest Finnigan TSQ 7000, Finnigan, Bremen, Germany). For further experiments, the peptide was first diluted to 100 μM in ethanol and then to 1 μM in L-PBS × 0.005% (w/v) Tween 20.

Capture ELISA

High-bind 96-well microtiter plates (Corning, http://www.corning.com) were coated overnight at 4 °C with 75 μl per well of 150 ng ml^{-1} anti-2,4-D antibody in L-PBS. After three washing steps using 300 μl of D-PBS × 0.05% (w/v) Tween 20 (D-PBST), the plates were blocked for 3–4 h at rt with 250 μl of 1% (w/v) casein in D-PBS, and again washed four times with D-PBST. Peptides 1, 2 and 3 were serially diluted in 50 μl of maleate buffer × 0.005% (w/v) Tween 20. After 2 h and 30 min, the plates were washed four times with D-PBST and incubated for 60 min at rt using 75 μl of 1 μg ml^{-1} SA-HRP in 1% (w/v) casein in D-PBS. The plates were washed six times with D-PBST and developed with 75 μl of tetramethylbenzidine. The colour development was terminated after 30 min by adding 125 μl of 1 M H_2SO_4, and the absorption was measured at $\lambda = 450$ nm using a microtiter plate reader. The software GraphPad Prism 5 was used to fit the data curves.

Renin assay

The proteolytic reaction of renin was carried out in a 96-well polypropylene microtiter plate (Corning, http://www.corning.com). Either a defined concentration of human renin or murine plasma samples were serially diluted in 50 μl per well of 10 nM peptide in maleate buffer × 0.005% (w/v) Tween 20. For inhibition studies, constant concentrations of pepstatin were added to the peptide solution. The sealed microtiter plate was incubated for either 90 min or 20 h at 37 °C. The proteolytic reaction was terminated by adding 50 μl of a 2 μM pepstatin solution and incubating on ice for 10 min. From each cavity, 75 μl of the peptide solution was transferred to a high-bind 96-well microtiter plate which beforehand had been coated with 75 μl of 150 ng ml^{-1} anti-2,4-D antibody, washed and blocked as described above. After 2 h and 30 min at rt, the plate was washed four times with D-PBST, and incubated for 60 min at rt using 75 μl of 1 μg ml^{-1} of horseradish peroxidase-labelled streptavidin in 1% (w/v) casein in D-PBS. The plates were washed six times with D-PBST and developed with 75 μl of tetramethylbenzidine. The colour development was terminated after 30 min by adding 125 μl of 1 M H_2SO_4, and the absorption

was measured at $\lambda = 450$ nm with a microtiter plate reader. The software GraphPad Prism 5 was used to fit the data curves.

Results and discussion

Design of renin substrates

The optimal design of a peptidic substrate for the protease renin foremost depends on a good cleavage site that is available from the N-terminal amino acid sequence of angiotensinogen.[13] We chose the first 15 N-terminal amino acids of angiotensinogen (DRVYIHPFHLVIHNE) for detecting the activity of human renin. It should be noted that there is no photophysical constraint on the length of the sequence, in contrast to *e.g.* the length of FRET substrates. Furthermore, the peptide must be labelled at the N- and C-terminus for detection in a sandwich ELISA format: one label is required for immobilizing the peptide on the surface of a microtiter plate and the other label for binding a detection system. The hapten 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with aminoundecanoic acid is an optimal N-terminal label for binding a peptide to a microtiter plate coated with an anti-2,4-D-antibody (clone E2/G2).[17,22] Biotin is chosen as the C-terminal label due to its extremely high binding affinity to streptavidin.[23] The detection system consists of a streptavidin–horseradish peroxidase conjugate that catalyzes a chromogenic reaction.

Positively charged peptides may bind non-specifically to microtiter plates, which can be attributed to cation–π interactions between the side chains of lysine, arginine or histidine and polystyrene.[16,24] This non-specific binding, however, must be avoided to be able to distinguish between cleaved and intact peptides after a proteolytic reaction. As the peptidic renin substrate (Peptide 1) has a net charge of +3, peptides flanked by either PEG (Peptide 2) or both PEG and D-glutamate (Peptide 3) were synthesized to compensate for non-specific binding of the core region (Fig. 1A). The peptides were diluted serially on antibody-coated as well as uncoated microtiter plates (Fig. 1B–D) (a) to optimize the concentration of the coating antibody and (b) to find the peptide concentration that yields a high antibody-mediated signal without non-specific surface binding. Non-specific binding to microtiter plates is evident at high peptide concentrations and increases the signal intensity in addition to antibody-mediated binding. Peptide 3 (Fig. 1D) displays the lowest non-specific binding, which can be well separated from antibody-mediated binding. In general, peptide concentrations below 5 nM did not result in non-specific binding. Thus, a constant peptide concentration of 5 nM was employed in the sandwich ELISA for detecting the proteolytic activity of renin.

Renin assay

Both renin and the peptidic substrate are free in solution (homogeneous assay) to avoid surface effects on the enzyme reaction. The proteolytic activity of renin separates the N- and C-terminal label of the peptide. With increasing renin activity in a sample, the ratio between intact and cleaved peptides decreases. Serial dilutions of renin in defined peptide solutions (10 nM) lead from samples that contain only cleaved peptides over mixtures to samples that contain only intact peptides. After transferring each sample to an antibody-coated microtiter plate, both cleaved and intact peptides are immobilized proportionally *via* their N-terminal 2,4-D label. An ELISA-signal, however, is generated only by the biotin label of the intact peptide. As the signal intensities in a well are affected by the experimental – in particular coating – conditions, the intact peptide was used as a reference signal to obtain ratiometric measurements that are essentially independent of absolute signal intensities.[25,26] The ratio of the signal after renin incubation to the reference signal thus defines the degree of proteolytic degradation.

For data analysis according to Michaelis–Menten kinetics, usually steady-state conditions are given by an excess of substrate over enzyme ([S] ≫ [E]). Due to the very high sensitivity of peptide detection using the sandwich ELISA, peptide concentrations lower than 10 nM can be detected. The renin concentration, by contrast, must be high enough for a detectable turnover (up to 12.5 nM, Fig. 2), which seems to violate the

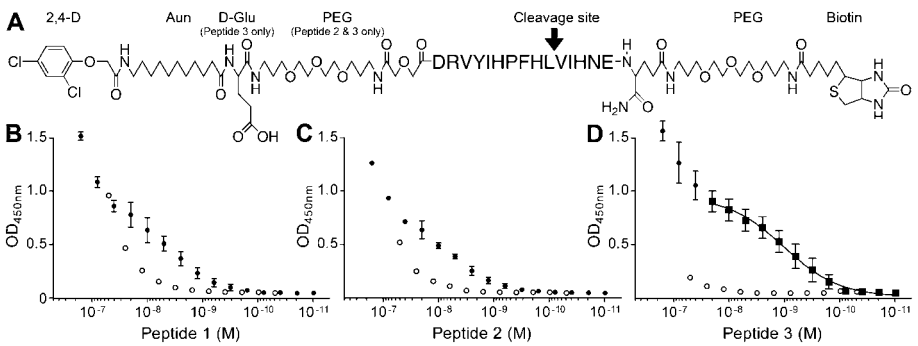

Fig. 1 (A) Peptide 1 consists of the N-terminal label 2,4-dichlorophenoxyacetic acid (2,4-D), aminoundecanoic acid (Aun), the core peptide sequence (15 amino acids) including the cleavage site for renin, a short polyethylene glycol (PEG), and the C-terminal label biotin. The sequence of Peptide 2 is extended by a short PEG, and Peptide 3 by both D-glutamic acid (D-Glu) and a short PEG. (B–D) Peptides are diluted serially on either an uncoated microtiter plate (empty circles) or a microtiter plate coated with 150 ng ml^{-1} of anti-2,4-D-antibody (full circles). (D) The optical densities (OD_c) obtained at different peptide concentrations (c) without interference by non-specific binding (solid squares) are fitted to a logistic function: $OD_c = (OD_{max} - OD_{bg})/(1 + (c/EC_{50})^s) + OD_{bg}$, which yields the highest optical density ($OD_{max} = 0.95$), the background ($OD_{bg} = 0.01$), the half effective concentration ($EC_{50} = 1.0$ nM), and the slope at the inflection point ($s = 0.86$). Error bars indicate the standard deviation of at least three measurements.

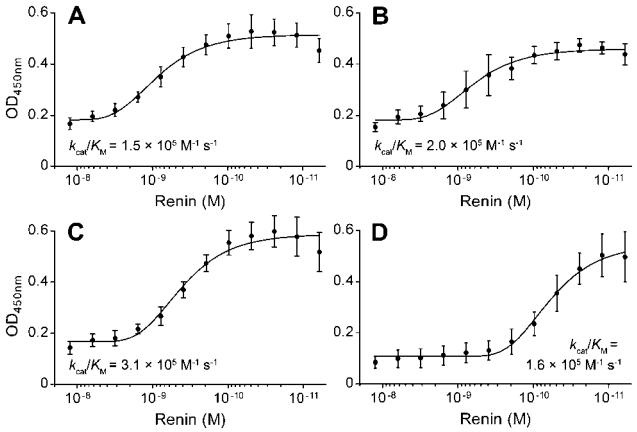

Fig. 2 Proteolytic degradation of Peptide 1 (A), Peptide 2 (B) and Peptide 3 (C and D) by human renin. Dilution series of renin were applied to the peptides in solution (10 nM) for either 90 min (A–C) or 20 h (D) at 37 °C. After terminating the enzyme reaction by a two-fold concentrated inhibitor solution, the peptides (5 nM) were detected using a sandwich ELISA. The data (four measurements; the error bars indicate the standard deviation) were fitted to eqn (2). The strongest signal response of OD_{450nm} to varying renin concentrations is observed in the test-midpoint, which is used to calculate k_{cat}/K_M.

steady-state approximation. Segel pointed out, however, that the steady-state approximation also holds with $[S] + K_M \gg [E]$.[27] As K_M of good peptidic renin substrates lies in the range of 0.5 to 1 μM,[13] the steady-state approximation is valid for the new renin assay.

The integrated Michaelis–Menten equation describes the progress of the enzyme reaction:

$$K_M \times \ln \frac{[S]_0}{[S]} + [S]_0 - [S] = k_{cat} \times [E] \times t \quad (1)$$

where [S] is the substrate concentration (0 indicates the initial substrate concentration/intact peptide), [E] the enzyme concentration, t the time, and K_M and k_{cat} are the kinetic constants. [S] can be replaced by the background corrected optical density (OD) of the ELISA measurement and the reference signal $[S]_0$ is given by OD_{max}.

Under the conditions used for the renin assay ($[S] \ll K_M$), eqn (1) reduces to a pseudo-first order reaction:

$$OD = (OD_{max} - OD_{bg}) \times e^{\left(-t[E]\frac{k_{cat}}{K_M}\right)} + OD_{bg} \quad (2)$$

which yields the second order rate constant k_{cat}/K_M as a regression parameter. k_{cat}/K_M is the most accurate kinetic constant for characterizing a protease's specificity for a substrate.[28] While it is common to measure the reaction progress after various time intervals, eqn (2) also allows for varying the enzyme concentration at constant incubation times. This approach is more favourable when carrying out kinetic experiments in a highly parallel way using a sandwich ELISA.[16]

Incubating Peptide 1, 2 and 3 for 90 min with 10 nM to 10 pM of human renin resulted in a continuous transition in OD from completely degraded peptides (OD_{bg}) to intact peptides (OD_{max}) that was used to calculate k_{cat}/K_M according to eqn (2) (Fig. 2). Structural modifications in the flanking region of the 15 amino acid-long core peptide (see Fig. 1A) had some effect on k_{cat}/K_M. Peptide 3, which was the best candidate with respect to low non-specific binding, also yielded the highest k_{cat}/K_M of 308 000 M^{-1} s^{-1} (Fig. 2B). After a prolonged incubation time of 20 h, however, this value appeared to decrease to 159 000 M^{-1} s^{-1} (Fig. 2D), which can be attributed to a loss of enzyme activity. Using a relatively short and constant incubation time of 90 min instead of time series, thus, is a simple way to avoid a loss of enzyme activity.

Table 1 Renin assays based on synthetic substrates

Method	Sequence motif	k_{cat}/K_M (M^{-1} s^{-1})
ELISA-based renin assay[a]	DRVYIHPFHL|VIHNE(15 aa)	308 000
Radioimmunoassay[37]	DRVYIHPFHL|VIHN (14 aa)	250 000
HPLC[38]	DRVYIHPFHL|VIHS (14 aa)	180 000[b]
FRET(DNP/Amp)[13]	DRVYIHPFHL|VIH (13 aa)	181 000
FRET(DNP/Amp)[13]	KHPFHL|VIH (9 aa)	352 000
FRET(EDANS/DABCYL)[c,13]	IHPFHL|VIHT (10 aa)	268 000

[a] Present study. [b] Estimated from K_M = 8.4 μM and V_{max} = 11 μmol angiotensin I made per h per mg renin.[38] [c] Commercial substrate.

The double-labelled renin substrates employed in the ELISA-based assay yielded comparable results for k_{cat}/K_M as FRET-based substrates of similar length (Table 1). Paschalidou *et al.*[13] characterized the influence of the peptide length in FRET-based substrates on k_{cat}/K_M in detail. When they extended the peptide sequence one amino acid at a time, k_{cat}/K_M increased successively but an optimum was not reached with a length of 13 amino acids (181 000 M^{-1} s^{-1}). As the quenching efficiency decreases with the sixth power of the distance between the donor and acceptor, the FRET substrate comprising 13 amino acids resulted in a signal to background ratio of only five and thus was classified as rather insensitive. Longer peptides are in general not practical. To circumvent this length limitation, the amino acid sequence was modified in search of optimal shorter substrates (Table 1).

Analysis of plasma samples

As the new protease assay is robust even in raw intestinal fluids,[16,29] it was applied to analyzing the activity of renin in raw murine plasma. Experimental details are given in the ESI.† Mice were treated intraperitoneally with isoproterenol to stimulate the production of renin, and one hour later plasma from treated and untreated mice was collected.[6] Using a commercial radioimmunoassay and angiotensinogen as a substrate, it was shown that the renin activity was 100-fold higher in treated than in untreated mice. In the ELISA-based renin assay, however, both plasma samples showed the same activity on murine peptidic substrates, which can be explained by the nonspecific activity of other proteases than renin in plasma. The three-dimensional conformation of the large (50 kDa) natural substrate angiotensinogen protects the cleavage site from various other proteases present in blood. Here, we have presented experimental evidence for the non-specific activity of plasma on synthetic renin substrates that has been described only marginally in previous literature reports.[15,30]

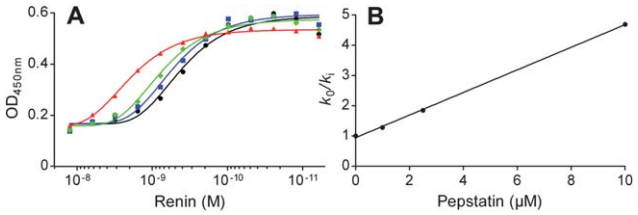

Fig. 3 Inhibition of human renin by pepstatin. (A) Renin was diluted serially on the peptide without inhibitor (black circles), and in the presence of 1 μM (blue squares), 2.5 μM (green diamonds) and 10 μM (red triangles) of pepstatin. (B) The rate constants were normalized according to eqn (5), and the slope of the linear regression yielded $K_i = 2.7$ μM ($r^2 = 0.9994$). Data points represent the mean of at least three measurements.

Inhibition of renin

Due to the key role of renin in the regulation of blood pressure, it is essential to identify potent renin inhibitors.[4,31,32] The renin assay can be adapted to inhibitor screening by diluting renin serially on a fixed mixture of peptidic substrate and competitive inhibitor, incubating for 90 min at 37 °C, and detecting the proteolytic reaction on an antibody-coated microtiter plate. The inhibition of renin can then be analyzed based on the integrated Michaelis–Menten equation for competitive inhibition:

$$K_M \times \left(1 + \frac{[I]}{K_i}\right) \times \ln \frac{[S]_0}{[S]} + [S]_0 - [S] = k_{cat} \times [E] \times t \qquad (3)$$

where [I] is the inhibitor concentration and K_i is the inhibition constant. Under pseudo-first order reaction conditions ($[S] \ll K_M$), the rate constant $k_0 = [E]\, k_{cat}/K_M$ of the uninhibited reaction in eqn (2) changes to the rate constant k_i of the inhibited reaction:

$$k_i = [E] \frac{k_{cat}}{K_M\left(1 + \frac{[I]}{K_i}\right)} \qquad (4)$$

Using various inhibitor concentrations and standardizing to the uninhibited reaction (k_0) yields a linear equation:

$$\frac{k_0}{k_i} = 1 + \frac{[I]}{K_i} \qquad (5)$$

Starting from a *y*-intercept of 1 without inhibitor, k_0/k_i increases linearly with the inhibitor concentration, and K_i can be obtained from the slope.

The microbial peptide pepstatin (isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid)[33] is a competitive inhibitor of renin[18,34] and other aspartyl proteases.[35] Pepstatin is a well-characterized transition state analog of aspartyl protease substrates that constitutes a good model system for the inhibition of renin. Using the ELISA-based renin assay, a K_i of 2.7 μM pepstatin was calculated for the inhibition of human renin (Fig. 3). The excellent linearity of k_0/k_i with the inhibitor concentration shows that it is sufficient to use only a single inhibitor concentration to determine K_i accurately if k_{cat}/K_M of the uninhibited reaction is known. Literature reports of K_i are also in the lower micromolar range, but vary because the inhibition by pepstatin is influenced by the pH, the substrate, and other experimental conditions. For example, when angiotensinogen was used as the substrate, the K_i was 1.4 μM pepstatin at pH 5.7, but 16 μM at pH 7.4.[19] When a synthetic tetradecapeptidic substrate was used at pH 6.5, a K_i of 15 μM was calculated.[20]

Conclusions

The variations in the determination of K_i among literature values highlight the need for well-defined experimental conditions to obtain comparable results from inhibitor screening. Here, we have presented a simple and robust kinetic analysis that yields excellent kinetic values for the activity of renin and its inhibitors. In contrast to the widely used FRET-substrates, the new renin analysis is not influenced by the absolute signal intensity, optical interference in renin samples, and the length of the renin substrates. The new kinetic analysis can be easily adapted to inhibitor screening of other medically important proteases. For example, the renin–pepstatin interaction can serve as a model for the inhibition of the aspartyl-protease from the human immunodeficiency virus (HIV),[36] which is a major drug target for the treatment of HIV.

Abbreviations

ACE	Angiotensin-converting enzyme;
AT_1 receptor	Angiotensin II receptor subtype-1;
Aun	Aminoundecanoic acid;
2,4-D	2,4-dichlorophenoxyacetic acid;
RAS	Renin–angiotensin system.

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