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The first potent diphenyl phosphonate KLK4 inhibitors with unexpected binding kinetics

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KLK4 is a serine protease from the kallikrein family that is involved in cancer progression. The diphenyl phosphonate warhead is intended to bind irreversibly with serine proteases, but unexpectedly, very potent KLK4 diphenyl phosphonate inhibitors were discovered with reversible inhibition kinetics.

Introduction

Human tissue kallikrein 4 (KLK4) is a trypsin-like serine protease of 24.1 kDa which belongs to a 15-member family of closely related peptidases (kallikrein-related peptidases, or KLKs). KLK4 is expressed as a preproprotein of 254 amino acid (aa) residues, converted to a 228 aa proprotein, and activated to a 224 aa mature proteinase by a metalloprotease.[1] Strikingly, KLK4 is imperative for tooth development, particularly in the formation of enamel, which also depends on MMP-20.[2,3] In adults, KLK4 is mainly expressed in prostate epithelial cells. Even though its physiological role in the prostate is not extensively studied, it was recently demonstrated that KLK4 is up-regulated in prostate cancer[4,5] and is related to a poor prognosis.[6] Like other KLKs, KLK4 has extracellular hydrolytic activity, such as activating and/or degrading matrix proteins,[7] cancer associated proteins,[8] proteases,[9] and signalling receptors.[10] In both patho- and physiological conditions, KLK4 expression is regulated by steroid hormones in a tissue-specific manner. The androgens influence its expression in prostate and breast cancer cells,[11,12] whereas estrogen causes expression in endometrial and ovarian cancer cells.[13] Notably, KLK4 has the capacity to activate the urokinase-type plasminogen activator (pro-uPA), as well as to modulate the activity of its receptor (uPAR), both of which play a significant role in metastatic cancers.[10,14] All these findings suggest that KLK4 can find application both as cancer biomarker,[15,16] and therapeutic target.[8,12]

To date, a number of natural inhibitors (serine protease inhibitors, or serpins) with physiological implication in KLK4 activity regulation are known, but only a small group of non-natural inhibitors has been discovered.[17] Among these we can find peptides like sunflower trypsin inhibitor and its analogues.[18] Currently, no small-molecules are known as KLK4 inhibitors. Since its predominant role in the development and progression of prostate cancer [19] and chemoresistance in ovarian cancer,[11] KLK4 emerges as a suitable target for the development of antitumor agents.

Several diphenyl phosphonate compounds have been identified as potent, irreversible serine protease inhibitors.[20-36] Interesting examples are a uPA inhibitor reported by Joossens et al. (compound 8b, k_{app} = 1x10^6 M^{-1}s^{-1})[21], an elastase inhibitor by Winiarski et al. (compound 61, k_{app} = 2x10^6 M^{-1}s^{-1})[34], a subtilisin inhibitor by Pietrusewicz et al. (compound 43, k_{app} = 1x10^5 M^{-1}s^{-1})[35], a GluC inhibitor by Burcharcka et al. (compound 8a, k_{app} = 8x10^3 M^{-1}s^{-1})[36], a DPP IV inhibitor by Belyaev et al. (compound 11e, k_{app} = 2x10^3 M^{-1}s^{-1})[31], and a DPP8 inhibitor by Van der Veken et al. (compound 2e, k_{app} = 4x10^3 M^{-1}s^{-1})[33]. Here we describe a series of diphenyl phosphonate compounds, as the first small molecule inhibitors of KLK4 (Table 1). KLK4 belongs to the S1 protease family with a preference for cleaving amide substrates following Arg > Lys > Tyr at the P1 positions.[37,38] Traditionally a peptidic tail is added to that position to obtain more potent and selective diphenyl phosphonate inhibitors. Nevertheless we published the design and evaluation of very potent and selective non-peptidic diphenyl phosphonate uPA inhibitors[21] lacking a peptidic tail. We designed these small compounds around the preferred P1 position since it drove the diphenyl phosphonate warhead most optimally in the direction of the catalytically active serine, followed by the formation of a covalent bond between the phosphorous atom of the warhead and the serine alcohol of the enzyme. The binding mechanism of the diphenyl phosphonate warhead is explained in *Figure 1*. Based on the same rationale we made a selection of Arg, Lys and Tyr diphenyl phosphonate mimetics and evaluated them for KLK4 inhibition.[20-22, 39-40, 42] This study will show that screening of a focussed library of compounds designed for irreversible inhibition can generate surprising results. Specific attention should be given to the kinetic binding mechanism, even if the IC_{50} values show strong binding to the target. Because selectivity issues are generally observed with protease inhibitors, we decided to test the compounds against a broader protease panel.

Results and discussions

Compounds **1a-f,h** and **2a-b** were synthesized as previously described (see Scheme 1 and 2, Supporting Information).[21, 24, 25, 39, 41] Compounds **1a-i** and **2a-b** were tested in enzymatic assays to determine their ability to inhibit KLK4 as well as 8

other related trypsin-like serine proteases and 1 serine hydrolase.

Our compound evaluation approach started with the evaluation of enzyme inhibition at 3 inhibitor concentrations (250 µM, 2.5 µM and 25 nM), followed by the IC_{50} and progress curve determinations for compounds showing more than 50% inhibition at 2.5 µM. Corresponding IC_{50} values were calculated using dose-response curves and are reported in *Table 1*. The 4 Parameter Logistic nonlinear regression model was used for curve-fitting analysis (Grafit.v7 (erithacus.com)). This model does not make any assumptions about the interaction and provides 4 parameters: a minimum asymptote, a maximum asymptote, the Hill slope describing the steepness of the curve, and the inflection point at a concentration defined as the IC_{50}. All available curves and equations can be found in the supplementary information. Most of the tested compounds exhibited excellent potency in inhibiting KLK4. In fact, compounds **1b**, **1e-h** and **2a-b** showed IC_{50} values in the sub-micromolar/nanomolar concentration range (IC_{50} range 0.003-0.250 µM).

The most potent KLK4 inhibitor within this series was compound **1e**, which showed an IC_{50} value of 3.4 nM. We recently published compounds **2a** and **2b** which were generated during the validation of a MSAS approach to obtain uPA inhibitors.[42] Interestingly they also show some KLK4 inhibition, although the alpha amino substitutent is not present. This emphasizes the strength of the P1 side chain as a directing group for the diphenyl phosphonate interaction with the catalytic serine of the protease.

In particular, all the active compounds are characterized by the presence of a guanidine or amidine function, as expected from the substrate selectivity of the enzyme. As mentioned before, KLK4 is able to recognize and cleave the peptide bonds of the substrate predominantly after an arginine residue, however it also cleaves after lysine or tyrosine at the P1 position of a substrate. Compounds characterized by aniline and phenol moieties in the P1 position (**1c, 1d, 1i**) did not display potent KLK4 inhibition. The most potent KLK4 inhibitor of this series (**1e**) also shows a nice selectivity. The selectivity ratio (IC_{50} enzyme/IC_{50} KLK4) is about 70 for KLK2 and KLK1 and is more than 300 for the other enzymes.

The diphenyl phosphonate derivatives are known as irreversible inhibitors of serine proteases, hence, we performed kinetic studies to determine the inhibition mechanism of selected compounds with significant KLK4 inhibition at concentrations lower than 2.5 µM. Based on the obtained progress curves we determined the type of binding kinetics. (Figure 2A) For the most interesting compounds we confirmed the type of

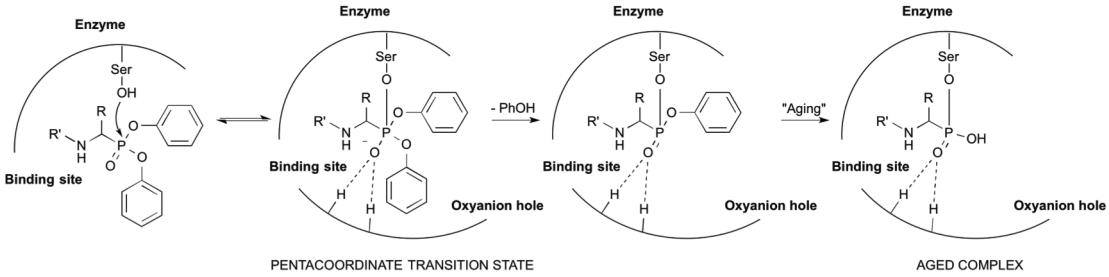

Figure 1. Binding mechanism of diphenyl phosphonates. The warhead is directed towards the active serine followed by a pentacoordinate transition state. After release of one phenol a covalent bond is formed. The second phenol can be released during a so called aging process by the influence of a water molecule

inhibition by a dilution experiment. *(Figure 2B)*. Unexpectedly the progress curve of **1e** shows a reversible binding type for KLK4. (*Figure 2*) In these experiments, enzyme and inhibitor were incubated at 37°C upon addition of the substrate (Boc-Val-Pro-Arg-AMC). The conversion of the substrate to the fluorescent aminomethyl-coumarin is monitored and the results are expressed as relative fluorescence unit (RFU) against time. A typical irreversible binder shows progress curves which show final velocities equal to zero and these curves are described by the following equation: $v_t = v_o e^{-kobs*t}$. However, in the reversible inhibition curve, (**1e**) the steady-state rate is different from zero since the enzyme is not completely blocked and conversion of the substrate continues after the time dependent inhibitor binding step. This curve is characterised by three phases: an initial phase that extrapolates to time =0 with slope v_0, final phase with a slope equal to v_s, and an exponential phase that connects the two linear phases with a pseudo-first order rate constant of k_{obs}. All equations used for fitting the progress curves are described in Lambeir et al.[43] The progress curve of **1e** is consistent with a slow reversible type of inhibition. Fitting the progress curve of this reaction gives a value of $k_{obs} = k_{off} + k_{on}[I]$ and thus allows to estimate k_{off} when k_{on} and [I] are known. In the experiment of Figure 2B the rate of dissociation is comparable with the rate of binding at the lowest inhibitor concentrations used, so there is no need to hypothesize that a covalent adduct is formed and subsequently hydrolyzed over the course of hours.

We have performed progress curves and dilution experiments for all compounds showing more than 50% inhibition at 2.5 µM. (see supplementary data). Compounds **1b**, **1e**, **1f**, **1g**, **1h**, **2a** and **2b** all show a reversible type of binding. This study suggests that in the case of KLK4 inhibition, the presence of the diphenyl phosphonate warhead combined with its optimal P1 fragment is not sufficient to guarantee an irreversible binding.

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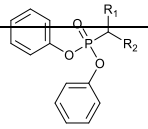
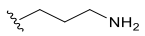
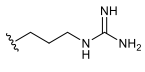
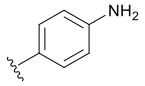
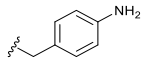
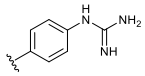
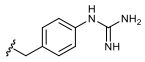
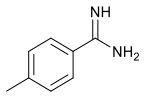
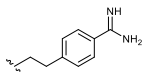
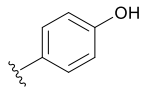
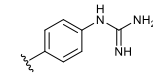
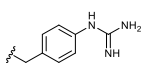
Table 1 Biological evaluation of KLK4 inhibitors **1a-i** and **2a-b**



Cpd	R_1	R2	KLK4 Type of binding	IC_{50} (μM)									
				KLK4	KLK2	KLK1	KLK8	uPA	tPA	Thrombin	Plasmin	FXa	AChE
1a†		NH-Cbz	ND	>2.5	>2.5	>10	>10	>250†	>2.5†	>2.5†	>250†	>250†	>10
1b†		NH-Cbz	Rev	0.04	>2.5	0.82	0.125	0.84**†	44†	0.8*†	30†	>10†	>10
1c¥		NH-Cbz	ND	>10	>2.5	>10	>10	>10	>10	>10	>10	>10	>10
1d†		NH-Cbz	ND	>10	>10	>10	>10	19.80	32	62	/	>10	/
1e¥		NH-Cbz	Rev	0.0034	0.22*	0.24*	1.16	2.70†	250†	250†	4.90†	3.60†	>10
1f†		NH-Cbz	Rev	0.012	0.53*	>10	0.17*	0.007**†	12†	2.40*†	3†	>10†	>20
1g†		NH-Cbz	Rev	0.041	>2.5	>2.5	0.79**	>2.5†	>2.5†	0.196*†	>2.5†	>2.5†	>10
1h†		NH-Cbz	Rev	0.015	0.72*	1.37	5	0.90**	68†	17†	6.20†	13.7†	>20
1i€		NH-Cbz	ND	>10	>2.5	0.086**	>10	>10	>10	>10	>10	>10	>10
2a§		H	Rev	0.25	2.44**	>2.5	1.44*	1.40*§	>10	>10	>10	>10	>10
2b§		H	Rev	0.08	3.02**	>2.5	0.17*	0.0079**§	>10	>10	>10	>10	>10

ND: not determined because IC50 above 2.5 μM; Rev: Reversible binding; *Reversible binding, **Irreversible binding. Standard deviation available in supplementory information. Synthesis or datapoints also reported in † ref 21, ¥ ref 25, € ref 39, § ref. 42,

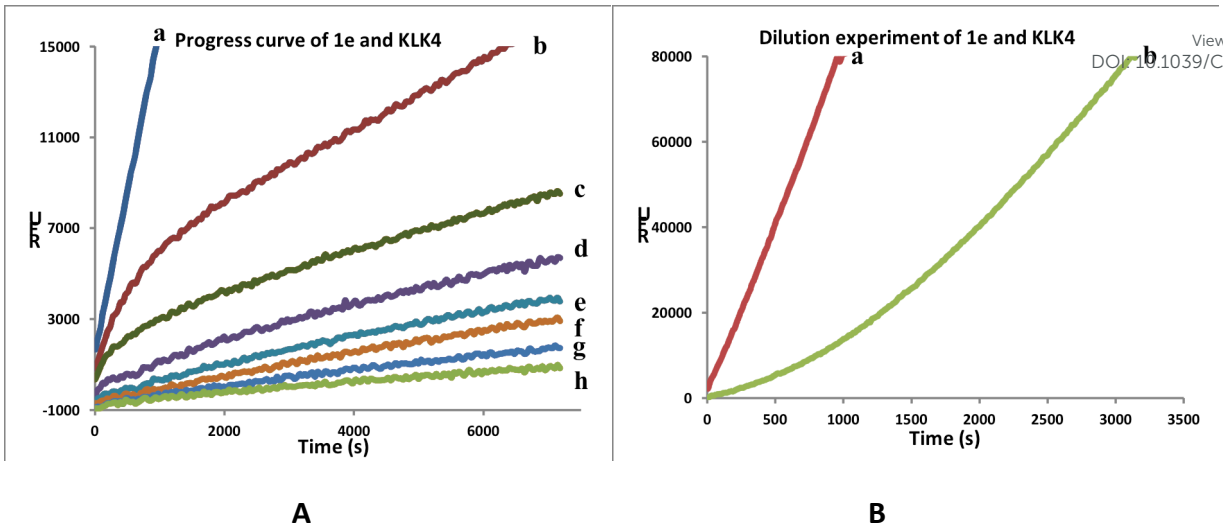


Figure 2. A: Progress curve of compound **1e** in the presence of KLK4. Each curve represents a different concentration of compound **1e** ((a) 0 µM, (b) 3 nM, (c) 6 nM, (d) 13 nM, (e) 25 nM, (f) 50 nM, (g) 100 nM, (h) 200 nM). The curves represent a reversible binding mechanism with slow type binding characteristics. **B**: This graph represents the results of a dilution experiment ((a) No inhibitor, (b) in the presence of **1e**). Compound **1e** was incubated at 300 nM with an enzyme concentration 100 times higher than within the IC_{50} assay. After 15 minutes the mixture was 100 times dilutes with buffer. If compound **1e** was an irreversible inhibitor the enzyme activity would be completely inhibited also after dilution (no increase

mode of the inhibitor to the enzyme. In the case of the here reported KLK4 inhibitors it can be hypothesised that upon interaction of the compounds with enzyme's P1 pocket, the phosphonate esters may not be properly positioned for a nucleophilic attack by the serine residue. We can state that the reason should not be found in the chemical properties such as stability or lower reactivity of the warhead, because compounds **1b**, **1f**, **1g**, **1h**, **2a** and **2b** show irreversible inhibition for uPA, KLK8 or KLK2. Detailed information can be found in Table 1 and the supplementary information. Compound **1g** is a promising hit candidate but shows strong inhibition of thrombin. This could cause side effects related to interference with the coagulation cascade, which needs to be avoided. Nevertheless, also for thrombin the determination of the binding characteristics is important. Reversible or irreversible inhibition of thrombin could lead to a different interpretation of the safety risk. We conclude that the ideal P1 substitution is not yet found to obtain potent, selective and irreversible KLK4 inhibitors. Alternatively, additional modifications such as modifications of the alpha amino substituents or replacements of the phenyl esters may be necessary to obtain selective irreversible KLK4 binders. Experiments are currently in progress to identify improved compounds which will be presented later in combination with their biological results

Conclusions

For the first time, we report potent small molecule KLK4 inhibitors. A set of compounds from a focused library designed for irreversible inhibition was tested in a KLK4 inhibition assay. From this preliminary study, five compounds (**1d-h)** emerged as good inhibitors with IC_{50} values in the range of sub-micromolar/nanomolar concentrations. Compound **1e** is a single digit nanomolar inhibitor of KLK4 with at least a 70-fold selectivity in a panel of highly homologous proteases. Surprisingly **1e** showed reversible binding kinetics. This study demonstrates that the type of inhibition should always be carefully investigated, even if potent IC_{50} values are obtained with compounds designed to be irreversible. These data represent a good starting point to develop selective KLK4 inhibitors. Irreversibility is a desirable characteristic fo diphenyl phosphonate inhibitors and a necessary prerequisite to develop activity-based probes to validate KLK4 as a target and/or biomarker in cancer. Further results in this area will be reported in due course.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1. N. Beaufort, K. Plaza, D. Utzschneider, A. Schwarz, J. M. Burkhart, S. Creutzburg, M. Debela, M. Schmitt, C. Ries, and V. Magdolen, *Biol. Chem.*, 2010, **391**, 581-587.
2. J. P. Simmer, M. Fukae, T. Tanabe, Y. Yamakoshi, T. Uchida, J. Xue, H. C. Margolis, M. Shimizu, B. C. DeHart, C. C. Hu, and J. D. Bartlett, *J. Dent. Res.*, 1998, **77**, 377-386.
3. P. S. Nelson, L. Gan, C. Ferguson, P. Moss, R. Gelinas, L. Hood, and K. Wang, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 3114–3119.
4. T. L. Veveris-Lowe, M. G. Lawrence, R. L. Collard, L. Bui, A. C. Herington, D. L. Nicol, J. A. Clements, *Endocr. Relat. Cancer*, 2005, **12**, 631–643.
5. L. Seiz, M. Kotzsch, N. I. Grebenchtchikov, A. J. Geurts-Moespot, S. Fuessel, P. Goettig, A. Gkazepis, M. P. Wirth, M. Schmitt, A. Lossnitzer, F. Sweep, and V. Magdolen, *Biol Chem,* 2010, **391**, 391-401.
6. C. V Obiezu, A. Scorilas, D. Katsaros, M. Massobrio, G. M. Yousef, S. Fracchioli, I. A. Rigault, D. Longrais, and R. Arisio, *Clin. Cancer Res.*, 2001, **4**, 2380–2386.
7. C. E. Smith, A. S. Richardson, Y. Hu, J. D. Bartlett, J. C.-C. Hu, and J. P. Simmer, *J. Biol. Chem.*, 2011, **286**, 18149–18160.
8. Y. Dong, D. Loessner, H. Irving-Rodgers, A. Obermair, J. L. Nicklin, and J. A. Clements, *Clin. Exp. Metastasis*, 2014, **31**, 135–147.
9. T. K. Takayama, B. A. McMullen, P. S. Nelson, M. Matsumura, and K. Fujikawa, *Biochemistry*, 2001, **40**, 15341–15348.
10. K. Oikonomopoulou, K. K. Hansen, M. Saifeddine, I. Tea, M. Blaber, S. I. Blaber, I. Scarisbrick, P. Andrade-Gordon, G. S. Cottrell, N. W. Bunnett, E. P. Diamandis, and M. D. Hollenberg, *J. Biol. Chem.*, 2006, **281**, 32095–32112.
11. J. Lai, S. A. Myers, M. G. Lawrence, D. M. Odorico, and J. A. Clements, *Mol. Cancer Res.*, 2009, **7**, 129–141.
12. Y. Jin, S. Qu, M. Tesikova, L. Wang, A. Kristian, G. M. Mælandsmo, H. Kong, T. Zhang, C. Jerónimo, M. R. Teixeira, E. Yuca, I. Tekedereli, K. Gorgulu, N. Alpay, A. K. Sood, G. Lopez-Berestein, H. E. Danielsen, B. Ozpolat, and F. Saatcioglu, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, E2572–E2581.
13. Y. Dong, C. Stephens, C. Walpole, J. E. Swedberg, G. M. Boyle, P. G. Parsons, M. A. McGuckin, J. M. Harris, and J. A. Clements, *PLoS One*, 2013, **8**, e57056.
14. N. Beaufort, M. Debela, S. Creutzburg, J. Kellermann, W. Bode, M. Schmitt, D Pidard, and V. Magdolen, *Biol. Chem.*, 2006, **387**, 217-222.
15. G. Sardana and E. P. Diamandis, *Clin. Biochem.*, 2009, **42**, 1483-1486.
16. M. Schmitt, V. Magdolen, F. Yang, M. Kiechle, J. Bayani, G. M. Yousef, A. Scorilas,, E. P. Diamandis, and J. Dorn, *Radiol. Oncol.*, 2013, *47*, 319-329.
17. P. Goettig, V. Magdolen, and H. Brandstetter, *Biochimie*, 2010, **92**, 1546–1567.
18. J. E. Swedberg, L. V Nigon, J. C. Reid, S. J. de Veer, C. M. Walpole, C. R. Stephens, T. P. Walsh, T. K. Takayama, J. D. Hooper, J. A. Clements, A. M. Buckle, and J. M. Harris, *Chem. Biol.*, 2009, **16**, 633–643.
19. T. I. Klokk, A. Kilander, Z. Xi, H. Waehre, B. Risberg, H. E. Danielsen, and F. Saatcioglu, *Cancer Res.*, 2007, **67**, 5221–5530.
20. J. Joossens, P. Van der Veken, A. M. Lambeir, K. Augustyns, and A. Haemers, *J. Med. Chem.*, 2004, **47**, 2411–2413.
21. J. Joossens, O. M. Ali, I. El-Sayed, G. Surpateanu, P. Van der Veken A.-M. Lambeir, B. Setyono-Han, J. A. Foekens, A. Schneider, W. Schmalix, A. Haemers, and K. Augustyns, *J. Med. Chem.*, 2007, **50**, 6638–6646.
22. J. Joossens, P. Van der Veken, G. Surpateanu, A.-M. Lambeir, I. El Sayed, O. M. Ali, K. Augustyns, and A. Haemers, *J. Med. Chem.*, 2006, **49**, 5785–5793.
23. Y. Nishiyama, H. Taguchi, J. Luo, Y. Zhou, G. Burr, S. Karle, and S. Paul, *Arch. Biochem. Biophys.*, 2002, **402**, 281–288.
24. J. Oleksyszyn, B. Boduszek, C. M. Kam, and J. C. Powers, *J. Med Chem.*, 1994, **37**, 226–231.
25. M. Sieńczyk, A. Lesner, M. Wysocka, A. Legowska, E. Pietrusewicz, K. Rolka, and J. Oleksyszyn, *Bioorg. Med. Chem.*, 2008, **16**, 8863-8867.
26. M. Sieńczyk and J. Oleksyszyn, *Bioorg. Med. Chem. Lett.*, 2006, **16** 2886–2890.
27. B. Walker and J. F. Lynas, *Cell. Mol. Life Sci.*, 2001, **58**, 596–624.
28. A. M. Lambeir, M. Borloo, I. DeMeester, A. Belyaev, K. Augusty D. Hendriks, S. Scharpe and A. Haemers, *Biochimica Et Biophysica Acta-General Subjects,* 1996, **1290**, 76-82.
29. S. Korom, S. I. DeMeester, T. H. W. Stadlbauer, A. Chandraker, M. Schaub, M. H. Sayegh, A. Belyaev, A. Haemers, S. Scharpe, J. W and K.Weglinski, *Transplantation*, 1997, **63**, 1495-1500.
30. I. DeMeester, A. Belyaev, A. M. Lambeir, G. R. Y. DeMeyer, G. VanOsselaer, A. Haemers, and S. Scharpe, *Biochemical Pharmacology*, 1997, **54**, 173-179
31. A. Belyaev, X. M. Zhang, K. Augustyns, A. M. Lambeir, I. De Meester, I. Vedernikova, S. Scharpe, and A. Haemers, *J. Med. Chem.* 1999, **42**, 1041-1052
32. K. Senten, L. Daniels, P. Van der Veken, I. De Meester, A. M. Lambeir, S. Scharpe, A. Haemers, K. Augustyns, *J. Com. Chem.* 2003, **5**, 336-344.
33. P. Van der Veken, A. Soroka, I. Brandt, Y. S. Chen, M.B. Maes, A. M. Lambeir, X. Chen , A. Haemers, S. Scharpe, K. Augustyns, and I. De Meester, *J. Med. Chem.,* 2007, **50**, 5568-5570.
34. L. Winiarski, J. Oleksyszyn, M. Sienczyk, *J. Med. Chem*., 2012, **55** 6541-6553.
35. E. Pietrusewicz, M. Sienczyk, J. Oleksyszyn, *J. Enzyme Inhib. Med. Chem*., 2009, **24**, 1229-1236.
36. E. Burchacka, M. Skorenski, M. Sienczyk, J. Oleksyszyn, Bioorg *Med. Chem. Lett.,* 2013, **23**, 1412-1414
37. M. Matsumura, A. S. Bhatt, D. Andress, N. Clegg, T. K. Takayama C. S. Craik, and P. S. Nelson, *Prostate*, 2005, **62**, 1-13.
38. M. Debela, V. Magdolen, N. Schechter, M. Valachova, F. Lottspeich, C. S. Craik, Y. Choe, W. Bode, and P. Goettig, *J. Biol. Chem.*, 2006, **281**, 25678-25688.
39. C. Bergin, R. Hamilton, B. Walker, and B. J. Walker, *Chem. Commun. (Camb).*, 1996, **10**, 1155–1156.
40. S. Dosa, M. Stirnberg, V. Lülsdorff, D. Häußler, E. Maurer, and M Gütschow, *Bioorg. Med. Chem.*, 2012, **20**, 6489–6505.
41. S. D. Larsen, M. A.Connell, M. M. Cudahy, B. R. Evans, P. D. May M. D. Meglasson, T. J. O'Sullivan, H. J. Schostarez, J. C. Sih, F. G. Stevens, S. P. Tanis, C. M. Tegley, J. A. Tucker, V. A. Vaillancourt, T. J. Vidmar, W. Watt, and J. H. Yu, *J. Med. Chem.*, 2001, **44**, 1217–1230.
42. R. Gladysz, M. Cleenewerck, J. Joossens, A.-M. Lambeir, K Augustyns, P. Van der Veken, *ChemBioChem*, 2014, **15**, 2238-2247
43. A.-M. Lambeir, M Borloo, I. De Meester I, A. Belyaev, K. Augustyn ,D. Hendriks,S. Scharpé, A. Haemers. *Biochim Biophys Acta.* 1996 **1290**, 76-82.

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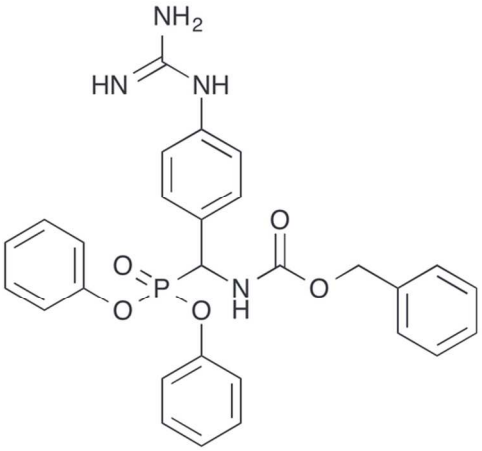

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We report the first highly potent and selective small-molecule KLK4 inhibitors, showing surprising reversible binding kinetics.

Graphical abstract
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