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Short Communication

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Insights into the activity control of the kallikrein-related peptidase 6: small-molecule modulators and allosterism

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Abstract: The activity of kallikrein-related peptidase 6 (KLK6) is deregulated in various diseases such as cancer and neurodegenerative diseases. KLK6 is thus considered as an attractive therapeutical target. In this short report, we depict some novel findings on the regulation of the KLK6 activity. Namely, we identified mechanism-based inhibitors (suicide substrates) from an in-house library of 6-substituted coumarin-3-carboxylate derivatives. In addition, a molecular dynamics study evidenced the allosteric behavior of KLK6 similar to that previously observed for some trypsin-like serine proteases. This allosteric behavior together with the coumarinic scaffold bring new opportunities for the design of KLK6 potent activity modulators, useful as therapeutics or activity-based probes.

Keywords: kallikrein-related peptidase 6; serine protease allostery; small-organic modulators; suicide substrate.

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The discovery of the kallikrein-related peptidase KLK6 was made almost concomitantly by different research groups in the mid-1990s. KLK6 belongs to the family of kallikrein-related peptidase family that consists of 15 (KLK1-15) secreted serine proteases and was isolated for the first time by Anisowicz and colleagues as ‘Protease M’ whose gene was overexpressed in breast and ovarian tumor tissues (Anisowicz et al., 1996). Furthermore, KLK6 was independently cloned by three laboratories as an important marker in neurodegenerative diseases. Yamashiro and coworkers have detected a very strong expression of its gene in the brain and named it ‘neurosin’ (Yamamura et al., 1997). Similar results have been reported by Little et al. who named this gene ‘zyme’ and highlighted its possible involvement in Alzheimer’s disease (Little et al., 1997). Finally, the human gene and also its murine orthologue were cloned by Scarisbrick and colleagues who named it ‘Myelencephalon-specific protease (MSP)’ (Bernett et al., 2002). The sequencing of the human genome and particularly of the kallikrein locus, showed that ‘Protease M’, ‘Zyme’, ‘Neurosin’ and ‘MSP’ correspond to the same enzyme, kallikrein-related peptidase 6 (KLK6, also hK6 in humans). KLK6 is widely expressed and is particularly abundant in the central nervous system (CNS) with a major distribution in the brainstem and spinal cord but also in hippocampus, frontal lobe, black substance, subthalamic nucleus and thalamus (Wang et al., 2008; Bayani and Diamandis, 2011; Scarisbrick and Blaber, 2013; Prassas et al., 2015). In the brain, KLK6 has been particularly studied for its involvement in neurodegenerative diseases and in certain tumors (Wang et al., 2008; Bayani and Diamandis, 2011; Scarisbrick and Blaber, 2013; Prassas et al., 2015). The first inhibitors of KLK6 have been identified in 2012 (Liang et al., 2012), using virtual screening and two series of derivatives were brought out, with either an amidinothiophene group or a *para*-amidobenzylamine group at the position P₁. More recently, a peptide inhibitor of KLK6 derived from its natural substrate, PAR-2

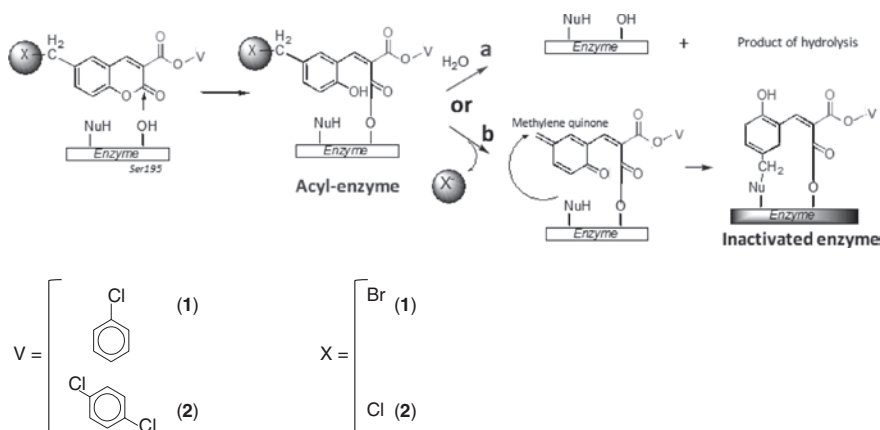


Figure 1: Structures of compounds **1** and **2** and their potential mechanism of inhibition of KLK6.

NuH, Nucleophile. Pathway a: transient inactivation by formation of a stable acyl-enzyme. Pathway b: suicide-type inactivation by the inhibitor acting as a suicide substrate.

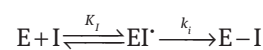
(proteinase activated receptor-2) was identified (Severino et al., 2015). However, no mechanisms were reported for these two categories of presumably reversible inhibitors. Thus, there is an important need to develop KLK6 inhibitors acting through mechanisms different from that previously described, like suicide substrates that may allow to fine tune selectivity.

Moreover, according to a newly emerging paradigm, the activity and specificity of serine proteases (especially for the trypsin-like family) suggest that the activity may be controlled by the equilibrium between an inactive mature (E^*) form and active mature (E) form (Di Cera, 2009a,b; Pozzi et al., 2012, 2013).

In this report, we contribute to the enlargement of the repertoire of KLK6 inhibitors by screening our previously reported coumarin library (Tan et al., 2015) and to the identification of mechanism-based inhibitors of KLK6. Moreover, we provided a molecular dynamics analysis of the KLK6 ‘apo-form’ that revealed an allosteric behavior with the identification of two enzymatic forms that rely on inactive and active mature conformations.

Coumarin derivatives display diverse pharmacological activities supporting their potential therapeutic use (as antitumor, anti-infective, anti-inflammatory and antioxidant agents) (Srikrishna et al., 2016). 6-Substituted coumarin-3-carboxylate derivatives were identified in our laboratory as inhibitors of the kallikrein-related peptidases KLK5, KLK7 and KLK14 involved in epidermis homeostasis and several skin diseases (Tan et al., 2015). 6-Substituted coumarin-3-carboxylate derivatives constitute remarkable non-peptidic protease inhibitors characterized by easy access to organic synthesis, high inhibitory potency and extreme sensitivity to structural variations at the 3- and 6-positions that influence not

only the specificity but also the inhibition mechanism of the targeted enzyme (Doucet et al., 1999). Inhibition of KLK5, KLK14 and matriptase by certain compounds was found to be mainly reversible, whereas KLK7 was always irreversibly inhibited through a suicide-type mechanism as shown in Figure 1. Several compounds acted selectively on KLK7, with IC_{50} values in the low micromolar and nanomolar ranges (Tan et al., 2015). By screening our whole library of 260 coumarinic compounds against KLK6, we identified two efficient inhibitors **1** and **2** (Figure 1) characterized by IC_{50} values of $3.1 \pm 0.2 \mu\text{M}$ and $0.9 \pm 0.1 \mu\text{M}$, respectively, after 15 min of treatment. To determine their mechanism of action, the dilution method was used. We showed that the activity of KLK6 previously inhibited at 80% was not recovered after a dilution by a factor of 100. Furthermore, to distinguish between a transient acyl-enzyme and an irreversible inhibition (Figure 1), we used the powerful nucleophile hydroxylamine at 0.5 M and pH 8.0 before determination of the remaining activity. After this hydroxylamine treatment, no reactivation was observed excluding a transient inhibition due the formation of a stable acyl-enzyme. Compounds **1** and **2** acted thus as irreversible suicide inhibitors following the minimum kinetic scheme:



where E and I are the free protease and the free inhibitor, EI is the kinetic chimera of the Michaelis complex and the acyl-enzyme and $E-I$ is the inactivated enzyme. The kinetic constant ratios k_i/K_i (efficacy indexes) were determined using the continuous method as described in Tan et al. (2015) (Figure 2A and B). We found that compound

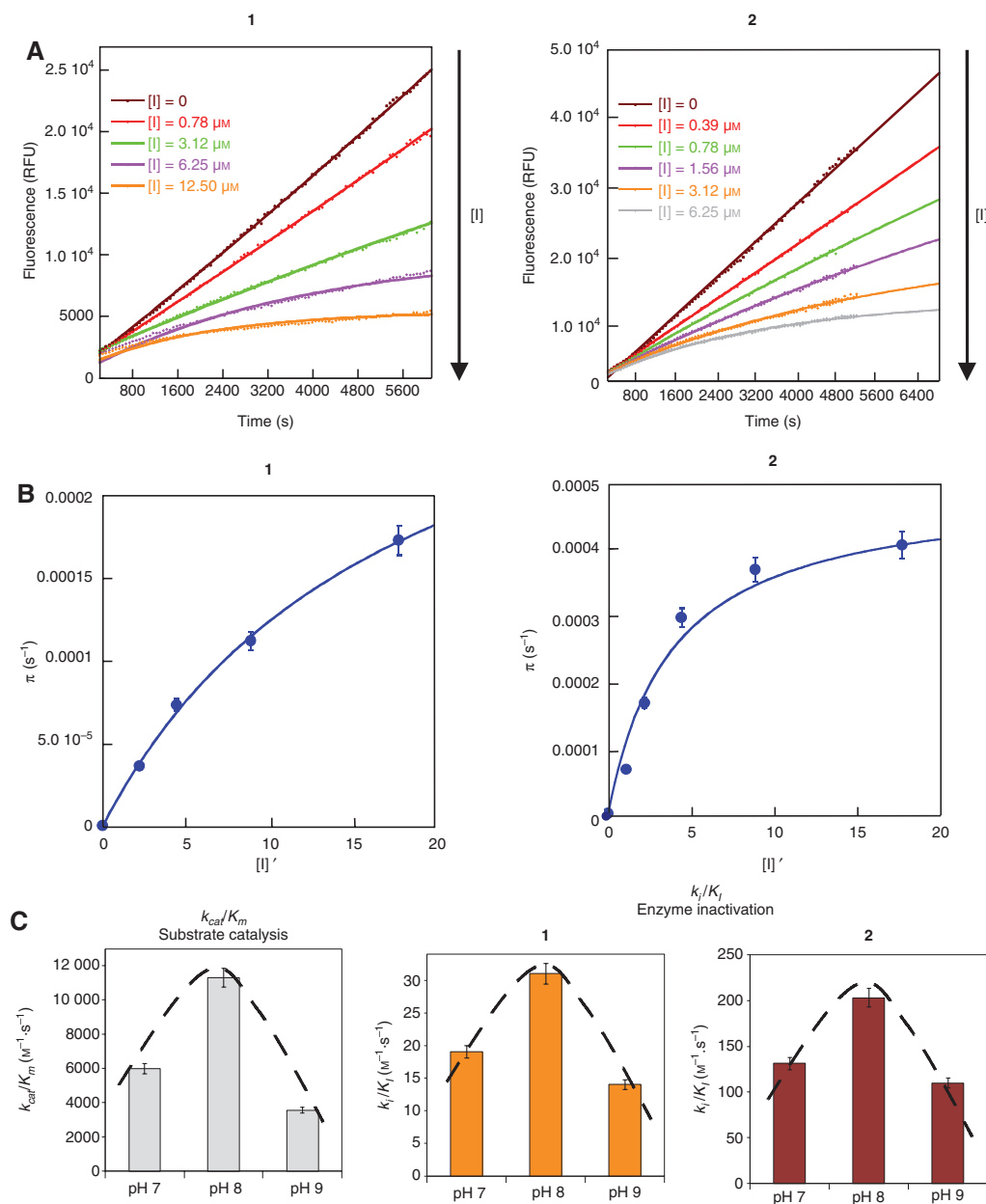


Figure 2: Inactivation of KLK6 by compounds **1** and **2** characterized by the continuous kinetic method at pH 7 and 37°C.

(A) Fluorescent product formed over time in the presence of different concentrations of compounds **1** and **2** (Active KLK6 was purchased from Abcam®, UK buffer: 50 mM Tris, 1 M citrate, 0.05% Brij-35; 100 μM Boc-QAR-AMC, Bachem, Switzerland). (B) Secondary graphs for the determination of k_i/K_i . The expressions of π and $[I]'$ are given in equation 1. Non-linear regression fits of the experimental data to equation 1 were performed using Kaleidagraph software. (C) Comparison between the pH profiles of substrate (Boc-QAR-AMC) hydrolysis into product (left) and inactivation of KLK6 by the suicide substrates **1** and **2** (right).

2 was a better inactivator of KLK6 than compound **1** with a k_i/K_i inactivation index of $131 \pm 13 \text{ M}^{-1} \cdot \text{s}^{-1}$ instead of $19 \pm 2 \text{ M}^{-1} \cdot \text{s}^{-1}$ for compound **2** (pH 7). It was also two-fold more efficient on KLK6 than on the KLK5, another trypsin-like kallikrein-related peptidase (Tan et al., 2015). To conclude, compound **2** constitutes a novel example of inhibitor acting irreversibly on KLK6 susceptible to be

optimized in order to develop tools as KLK6 activity-based probes. We also evaluated the influence of pH between 7 and 9 on inhibitory potency of compounds **1** and **2** (Figure 2C). The inactivation profile with pH followed the same progression than the enzymatic activity (k_{cat}/K_m). The optimum k_i/K_i index was found at pH 8 with $31 \text{ M}^{-1} \cdot \text{s}^{-1}$ for compound **1** and $203 \text{ M}^{-1} \cdot \text{s}^{-1}$ for compound **2**.

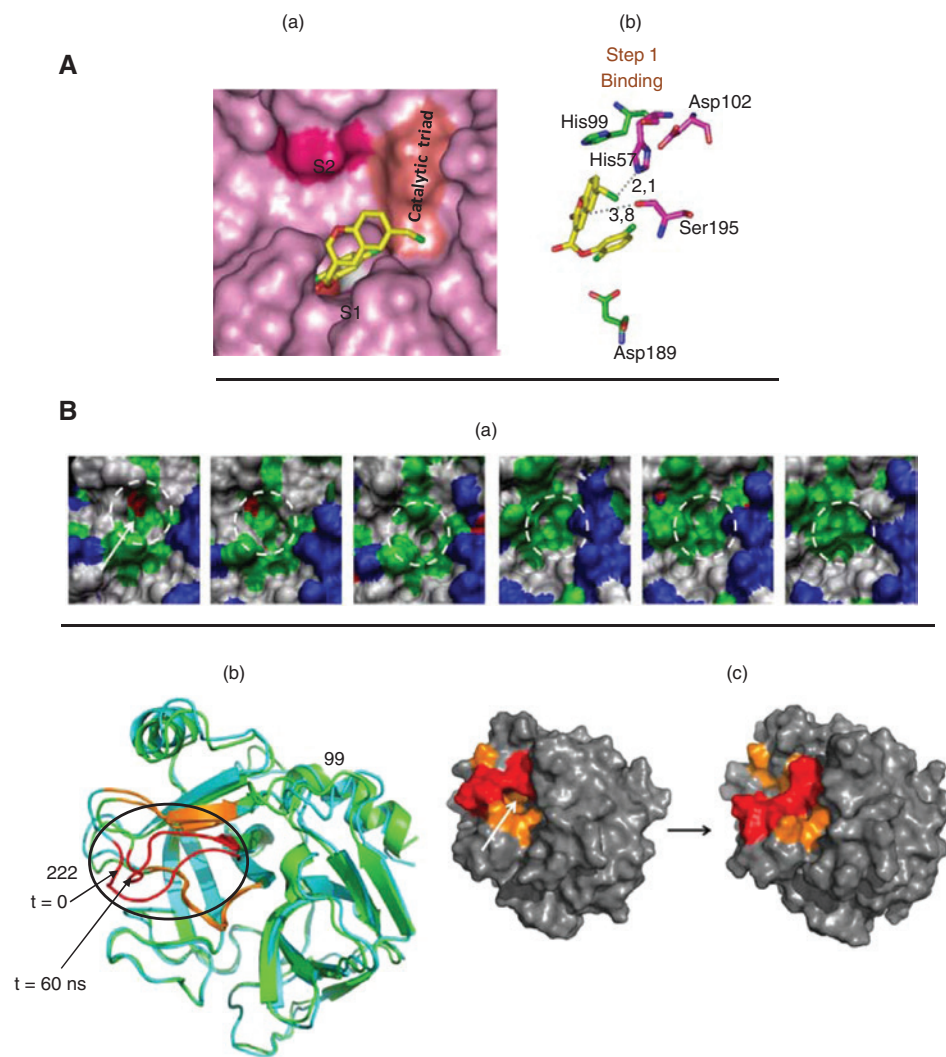


Figure 3: Structural insights into KLK6 activity modulation.

(A) Proposed structural basis for the inactivation of KLK6 by compound 2. (a) The *in silico* docking of compound 2 within the active site of KLK6 was performed using Molegro Virtual Docker. KLK6 is represented by pink surface and 2 by yellow sticks. The subsites S1, S2 and the catalytic triad are indicated. (b) Binding mode of 2 (yellow sticks) to active site residues (pink sticks). The distances (Ångstroms) in the binding mode between the lactone of coumarin ring the hydroxyl of the active serine 195 as well as the imidazole of the histidine 57 and the CH₂Cl group at X are indicated. (B) MD simulation of mature apo-KLK6, 60 ns using GROMACS 4.6.5. (a) Illustration of activity plasticity over the time, the broken-line circle materializes the active site, the catalytic serine Ser195 is shown in red. (b) Evolution of the global conformation of the apo-KLK6 in cartoon representation (blue, start of simulation; green, end of simulation). The strand and the loop of the S1 site are in orange, the loop 214–220 in red. (c) The ‘open’ active site of KLK6 (white arrow, left pose) is ‘closed’ at the end of the simulation (right pose). The KLK6 is shown in surface representation (gray). The residues of the S1 site are in orange, the residues 214–220 in red. S1 residues: 189–195, 214–220, 224–228. The volumes of the active site at the beginning and the end of the simulation are 92 Å³ and 43 Å³, respectively (Molegro Virtual Docker).

$$\text{Product} = \int_0^t v_i \cdot dt; v_i = v_0 * e^{-\pi t}; \pi = \frac{k_i * [I]'}{K_i + [I]'}$$

$$\text{where } [I]' = \frac{[I]}{1 + \frac{[S]}{K_m}} \quad (1)$$

The proposed mechanism-based inhibition was supported by molecular modeling performed using the

software Molegro Virtual Docker (Thomsen and Christensen, 2006) (Figure 3A). The analysis of the best pose for compound 2 bound to KLK6 active site revealed that the bi-substituted phenyl (2-Cl, 5-Cl) group was accommodated into the S1 subsite. The distance between the hydroxyl oxygen of Ser195 and the carbonyl carbon of the coumarin lactone was compatible with a nucleophilic attack by the catalytic serine that can lead to an

acyl-enzyme formation. The acyl-enzyme can follow two distinct pathways: (i) hydrolysis by a water molecule, allowing the regeneration of the catalytic site of KLK6; (ii) formation of an additional covalent bond involving a nucleophile (His57) after demasking of latent methylene quinone through elimination of the good leaving group Br⁻ (compound **1**) or Cl⁻ (compound **2**) (Figure 1). The distance (2.1 Å) observed between the CH₂Cl carbon and the imidazole group of His57 was compatible with such a reaction that can lead to the experimentally observed irreversible inhibition (Figure 3A).

In parallel to modeling studies, we performed molecular dynamics simulations (MD) that are very useful to predict and evaluate protein structural plasticity and stability, and understand enzymatic mechanisms particularly in the case of proteases. Only a few MD studies have been reported for kallikrein-related peptidases and they concerned KLK3, KLK5 and KLK7 complexed with inhibitors (Oliveira et al., 2014) or KLK4, KLK5 and KLK14 complexed to high efficient designed substrates (de Veer et al., 2012). We performed MD simulations of 60 ns in explicit solvent at 310 and 350 K using a KLK6-benzamidine complex (PDB code: 1L06) (Bennett et al., 2002) using GROMACS 4.6.5 (http://www.gromacs.org/About_Gromacs/) and AMBER99SB force field with a time step of 2 fs. Conformational behaviors of both complexed and apo-KLK6 (e.g. without ligand) have been analyzed applying GROMACS 5.1.2, VMD and PyMol softwares. No large amplitude movements within structural domains were noticed. However, in the absence of ligand, the active site volume surprisingly decreased significantly, from 92 Å³ to 43 Å³ within 20 ns (Figure 3B; see also the RMSD plots in the online Supplementary Material, Figures S1 and S2). When analyzing the structural details in particular at the vicinity of the active site, a reorientation of the 214–220 loop was observed and resulted in the obstruction of active site cavity by the 215–217 segment residues. In conclusion, the mature apo-form of KLK6 in the absence of ligand seems to be characterized by a closed inactive conformation (E* form), that prevents interaction with a ligand. Such a closed mature conformation implicating 215–217 segment was already reported for some serine proteases like thrombin but rarely for kallikrein-related peptidases. However, updated modalities of activity regulation within KLK family has been recently reported, through an exhaustive structural and kinetics study of KLK2 (Skala et al., 2014). A comparative structural analysis of 99-loop was performed and allowed to provide a rational classification. The 99-loop was identified as a key structural element to modulate KLK2 activity

particularly involved in zinc (Zn²⁺) binding. An interplay between the 99-, 148-, and 220-loops was reported. These regulating loops were suggested to fine-tune the equilibrium between open E and closed E* conformations of KLK2 through concerted conformational changes, allowing or preventing substrate access to the active site. These findings early allowed to extent the concept of conformational selection in trypsin-related proteases (E vs. E*). In contrast to KLK6, 99-loop is short without insertions suggesting distinct activity regulation (Gomis-Ruth et al., 2002; Skala et al., 2014). Our preliminary results suggest a novel hypothesis to better understand the activity regulation of KLK6 in accordance with the emerging concept of allostery of serine proteases. Ongoing biochemical studies in our lab particularly those using various chemical tools may allow to rationalize these *in silico* observations. In recent years, kinetic studies on thrombin (Lechtenberg et al., 2012), the key serine protease in the coagulation cascade, has demonstrated an unexpected plasticity of trypsin-like folding like thrombin. Fast kinetics experiments show that the E*-E conversion takes place on a time scale of less than 10 ms, while the zymogen-protease conversion evolves on a larger time scale (100–1000 ms) (Di Cera, 2009a,b). Hence, an analogous regulation of a reversible allosteric E*/E equilibrium in addition to zymogen to mature protease conversion could constitute innovative therapeutical strategies aiming to fine-tune active and inactive states of mature KLK6.

Taken together, we report the first KLK6 low-molecular-weight suicide substrates obtained from our coumarin library and provide data suggesting an allosteric behavior of KLK6 as previously reported for some other trypsin-like serine proteases like thrombin. We found that inhibitors **1** and **2** were devoid of cytotoxicity toward mouse cortical primary neurons up to 100 μM (data not shown). Finally, the 6-substituted coumarin-3-carboxylate structure represents a valuable scaffold that could serve for a platform for the design of activity-based probes to dissect out the KLK6 enzymatic activity in biological samples and pathophysiological processes such as CNS diseases.

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