# Novel inhibitory activity for serine protease inhibitor Kazal type-3 (Spink3) on human recombinant kallikreins

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Kallikrein-related peptidases (KLKs) are trypsin-like and chymotrypsin-like serine proteases which are expressed in several tissues. Their activity is tightly controlled by inhibitors including members of the serine protease Kazal-type (SPINK) family. These enzymes are promising targets for the treatment of skin desquamation, inflammation and cancer.

*Spink3* or caltrin I is expressed in mouse pancreas and males accessory glands and the resulting mature protein has been associated with different activities such as an inhibitor of trypsin and acrosin activity, calcium transport inhibitor in sperm and inhibitor of cell proliferation during embryogenesis. In this study we produced a soluble recombinant Spink3 from mouse seminal vesicle (rmSpink3) that inhibited the activity of human KLKs. Using FRET substrates, rmSpink3 exhibited a potent inhibitory activity against human KLK2, KLK3, KLK5 (KLKn) (Ki ranging from 260 to 1500 nM), and to a lesser extent against KLK6, KLK1 and KLK7 (Ki around 3000 nM). As shown by mass spectrometry analysis of rmSpink3 incubated with trypsin, the inhibitor was not truncated by the target enzyme. Based on the analysis in

25 silico of the expression of Spink3/SPINK1 and KLKs it is speculated that some KLKs may be natural targets

26 of Spink3/SPINK1, however experimental confirmation using both proteins from mouse or either from

27 human origin is needed.

28 This work shows rmSpink3 is a potent inhibitor of various human KLKs members suggesting the potential

29 of this molecule in the diagnosis/prevention of several human diseases.

30

31 ***1- Introduction***

32 Kazal-type protease inhibitors play important roles in many biological and physiological processes, such

33 as blood clotting, the immune response and reproduction [1].

34 Serine protease inhibitor Kazal type 3 (Spink3) is a mouse 6-kDa protein, also called P12 [2, 3], which was

35 initially described as an inhibitor of trypsin and acrosin [4]. The orthologue human protein, designated

36 SPINK1 or PSTI, was originally isolated from pancreas where its expression is constitutive [5-7] and

37 essential for the control of trypsin activity. SPINK1 was reported to differ from the other pancreatic

38 inhibitor BPTI because the former one lacks of inhibitory effect on chymotrypsin and pancreatic

39 kallikreins (KLKs) [7], however the effect was evaluated over porcine pancreatic kallikrein [8]. It has

40 been reported that mutations in *Spink1* are associated with hereditary pancreatitis [9]. Recent evidence

41 suggests that Spink3 is a multifunctional protein that functions as a growth factor for the regeneration

42 of acinar cells in pancreas as well as for the proliferation of various types of cells during early

43 embryogenesis [3, 7, 10, 11]. These are novel functions of this molecule apart from that already known

44 as a protease inhibitor. Spink3 has also been detected in the male accessory sexual glands and female

45 uterus under androgen and progesterone control, respectively. In this context, Spink3 secreted from the

46 mouse seminal vesicles and prostate, contributes to the seminal fluid [2]. MatureSpink3 attaches to the

47 sperm head [12], inhibits calcium uptake by spermatozoa [12, 13] and is involved in the sperm acrosome

48 reaction and zona binding [2, 14], independently of its trypsin inhibitory activity [12, 15]. However, the
49 target proteinase of Spink3 (if there is one) in organs other than the pancreas, remains unknown.
50 Kazal domain, which comprises most of the mature form of Spink3, is composed of 40–60 amino acid
51 residues that include six well-conserved cysteine residues capable of forming three intra-domain
52 disulfide bridges (cys 1–5, 2–4, 3–6). This assembly results in the characteristic three-dimensional
53 structure consisting of one α-helix surrounded by an adjacent ß-sheet and three loops of peptide
54 segments [16]. The inhibitory specificity of a Kazal domain varies with a different reactive P1 amino acid,
55 which is the second amino acid after the second Cys [1].The reactive loop harbors the specificity
56 determinant P1 amino acid and provides easy access to the active site of the cognate proteinase. The
57 Kazal domain acts as a substrate analogue that stoichiometrically and competitively binds to the active
58 site of the target proteinase forming a proteinase–proteinase inhibitor complex, much more stable than
59 the Michaelis enzyme–substrate complex [16].
60 KLKs are secretory serine proteases of small size (approximately 30 kDa). The human glandular *KLK* gene
61 locus on chromosome 19q13.4 is composed of 15 genes encoding serine proteases. Many members of
62 the human KLK family, in addition to KLK2 and KLK3 (prostate specific antigen, PSA), are promising
63 biomarkers for ovarian, prostate, and breast cancers since KLK proteinase expression, which in many
64 cases is under the control of steroid hormones, changes dramatically in a variety of malignant diseases
65 [17, 18]. KLK genes are widespread along mammalian [19] and non-mammalian [20] genomes indicating
66 their importance in the physiology. In mouse, the total number of glandular *Klk* genes is 37 of which 11
67 are pseudogenes. Interestingly, there are 12 genes that represent the orthologues of human *KLK* genes
68 (*KLK1, KLK4*–*KLK15*) being *KLK2* and *KLK*3 the only ones that do not have mouse orthologues [21, 22].
69 Given the pleiotropic roles of KLKs, both activators and inhibitors of KLK activities either from synthetic
70 or natural origin are of therapeutic interest [17, 23].

71 Many endogenous inhibitors are known to regulate the activity of KLKs forming complexes with plasma
72 inhibitors, including various serpins, such as α1-antitrypsin (α1-protease inhibitor), α1-antichymotrypsin
73 (ACT), protein C inhibitor (a.k.a. plasminogen activator inhibitor-3), plasminogen activator inhibitor-1,
74 antithrombin III, and α2-antiplasmin [24]. Furthermore, it has been proposed that inhibitory peptides
75 derived from Kazal type serine protease inhibitors may regulate KLK activity due to co-localization; that
76 is the case of KLKs and SPINK5 in the skin [25-27]. Another supporting evidence, is that both the tandem
77 localized *KLK* genes [28] and *SPINK1/Spink3* genes [29] are under transcriptional regulation by steroid
78 hormones. We have been working with Spink3 in the elucidation of its role on sperm physiology [12].
79 Our experience with Spink3 and the above mentioned evidences prompted us to speculate that Spink3
80 might in vitro regulate the activity of KLKs. , We have previously obtained a soluble recombinant GST-
81 Spink3 fusion protein which is devoided of trypsin inhibitory activity unless released from the GST
82 portion [12]. Although this recombinant Spink3 retained protease inhibitory activity, protein yield was
83 low which precluded scaling up. In this study, we developed a new construct that allowed the expression
84 of a version of recombinant mouse Spink3 (rmSpink3) with protease inhibitory activity and good yield.
85 This protein was an effective inhibitor on various humanKLKs of pharmacological interest.
86
87 ***2- Materials and methods***
88 *2.1- Heterologous expression of recombinant Spink3 (rmSpink3-His$_6$)*
89 A DNA fragment corresponding to mature Spink3 (nucleotides 158 to 331 which encoded a polypeptide
90 of 6.1 kDa starting from residue 24 to the stop codon) was previously cloned into pGEX-4T-3 expression
91 vector (GE) [12]. To facilitate subcloning of *Spink3* cDNA into the expression vector pET-24b(+)
92 (Novagen), the restriction sites NdeI and XhoI were added to the 5' end nucleotide sequences of Spink3-
93 Fw (5'-GG GAA TTC CAT ATG GCT AAG GTG ACT GG-3') and Spink3-Rev (5'-GGC CTC GAG GCA AGG CCC
94 ACC-TTT TC 3') primers, respectively. To obtain a fusion protein with a His-tag at the C-terminus (8,06

95 kDa), the stop codon of *mSpink3* was excluded in the Rev primer. The PCR reaction was carried out in a
96 mixture (20 µl) containing 1 x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 1 µM of primers, 15% DMSO,
97 300 ng pGEX-*Spink3* template and 2.5 U Taq DNA polymerase. The PCR amplification protocol was as
98 follows: 35 cycles of denaturation at 94°C for 1 min, primer annealing at 59° C for 1 min, and primer
99 extension at 72°C for 1 min. The PCR product of the expected size (204 bp) was agarose gel-purified
100 according to the MinElute purification kit (Qiagen).
101 The cDNA encoding mature Spink3 and pET -24b(+) expression vector were digested with *NdeI* and *XhoI*
102 (Promega) following the manufacturer's instructions and ligated with T4 DNA Ligase (Promega)
103 according to standard protocols. The generated construct, pET-24b (+) -*Spink3* which encoded
104 recombinant mouse Spink3 followed by a six-histidine His-tag at the C-terminus, (rmSpink3-$His_6$) was
105 then amplified in *E. coli* DH5α competent cells and the potential positive clones were confirmed by DNA
106 sequencing.
107
108 For overexpression of rmSpink3-$His_6$, *E. coli* Rosetta cells (Novagen) were transformed with the pET-24b
109 (+)-*Spink3* and the cells harboring the plasmid were grown overnight at 37 °C in Luria Bertani (2YT-LB)
110 medium (1% yeast extract, 2% tryptone, 0.5% NaCl) supplemented with kanamycin (50 µg/ml) and
111 chloramphenicol (35 µg/ml). This starting culture was used to inoculate (1/100) the same medium (50
112 ml) and the culture was grown at 37 °C under continuous shaking until the optical density at 600 nm
113 ($OD_{600}$) reached 0.4, then shifted to 18 °C and further incubated to an $OD_{600}$ ~0.6. Synthesis of rmSpink3
114 was induced by addition of 0.1 mM IPTG for 16 h at 18° C. Bacteria were harvested by centrifugation
115 (1700 x *g* for 8 min at 4° C), suspended in PBS (ICN tablet 2810305; 10 mM phosphate buffer pH 7.4, 137
116 mM NaCl, 2.7 mM KCl) containing 1 mg/ml lysozyme, 0.2% (v/v) Triton X-100 and 0.5 mM PMSF and
117 lysed by sonication (6 x 30 s 40 W, Cole Parmer 4710). The soluble (S) and insoluble (I) cell lysate

118 fractions were obtained by centrifugation (12000 xg for 25 min at 4 °C) and analyzed by SDS-PAGE (15%
119 w/v polyacrylamide gel) [30].
120
121 HiTrap IMAC HP affinity chromatography (28-4046-23 AA, GE) was applied for purification of rSpink3-
122 $His_6$ from the soluble fraction of induced cells. The column was loaded with 0.1 M $NiSO_4$, equilibrated
123 and washed with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole, pH 7.4).
124 Then, the soluble extract was loaded, washed and the recombinant protein was eluted with 20 mM
125 sodium phosphate, 0.5 M NaCl and 500 mM imidazole, pH 7.4. Purified rmSpink3 was concentrated by
126 ultrafiltration in YM-3000 membranes (Amicon) and the purity of the protein was evaluated by SDS-
127 PAGE (w/v 15% polyacrylamide gel). The identity of rmSpink3 was validated by Western blot.
128
129 *2.2- Western blot*
130 Proteins were fractionated on 15% (w/v) polyacrylamide gels and transferred to PVDF membranes for 2
131 h 80 mA in towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) using a TE 70 PWR transfer
132 equipment (Amersham). Nonspecific binding sites on the membrane were blocked with 3% skimmed
133 milk in TBS-N (10 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.1% ipegal CA630). Incubation with primary
134 antibody was performed for 1 h at 37 ºC with either anti-SPINK1 (1:4500, Sigma HPA027498) or anti-His
135 (1:3000, GE 27-4710-01) antibodies in blocking solution. After washing with TBS-N, the membrane was
136 incubated either with alkaline phosphatase-conjugated anti-rabbit IgG (1:10000, A8025 Sigma) or
137 alkaline phosphatase-conjugated anti-mouse IgG (1:5000, A3562 Sigma) and developed with 0.33 mg/ml
138 nitro-blue tetrazolium (NBT) and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline
139 phosphatase buffer (0.1M Tris pH 9.5; 0.1M NaCl and 5 mM MgCl2).The ability of the commercial anti-
140 SPINK1 (raised against an immunogenic sequence of 51 amino acids from SPINK1) to recognize
141 Spink3was previously confirmed [12].

142

143 *2.3- Determination of the kinetic parameters of KLK-Spink3 interaction*

144 Mature humanKLK1, KLK2, KLK3, KLK5, KLK6 and KLK7 were expressed and purified from a
145 baculovirus/insect cell line system as previously described [31]. Briefly, DNA fragments encoding the
146 mature form of KLKs were inserted in frame downstream the enterokinase propeptide nucleotide
147 sequence. To facilitate the purification of the recombinant proteins, a 6x His-tag codon was included at
148 the 3'-end of the enterokinase propeptide sequence. After purification by $Ni^{2+}$ affinity chromatography,
149 KLKs were activated by incubation (24 h) with enterokinase at 1:100 ratio enterokinase:pro-KLKs (w/w).
150 Hydrolysis of the enterokinase propeptide proceeded to apparent completion and mature KLKs were
151 purified by size exclusion chromatography. The final yield of purified mature KLKs was typically 20-25 mg
152 per liter of culture. Purity of mature KLKs was assessed by SDS-PAGE (w/v 15% polyacrylamide gel) and
153 Coomassie Brilliant Blue staining, N-terminal sequencing, and MALDI-TOF mass spectrometry. Protein
154 samples were about 98% homogeneous, with no visible degradation products and had the expected
155 mature N-terminus corresponding to the mature proteases.

156 All FRET peptides were obtained by solid-phase peptide synthesis as previously described [32, 33] and
157 using the Fmoc-procedure in an automated bench-top simultaneous multiple solid-phase peptide
158 synthesizer (PSSM-8 system from Shimadzu-Tokyo, Japan). The peptides were synthesized in TGR-resin
159 (loading 0.2 mmol/g) using HBTU/HOBt as coupling reagent and the cleavage o peptide resins was
160 accomplished with TFA:anisol:EDT:water (85:5:3:7). All peptides obtained were purified by semi-
161 preparative HPLC on an Econosil C-18 column. The molecular weight and purity (94% or higher) of
162 synthetized peptides were checked by MALDI-TOF mass spectrometry, using a Microflex LT mass
163 spectrometer (Bruker Daltonics, Billerica, USA). Stock solutions of the peptides were prepared in DMF
164 and the concentrations were measured by colorimetrically determination of the 2,4-dinitrophenyl group
165 (molar extinction coefficient of 17,300 M-1 cm-1 at 365 nm) [34]

166 To determine kinetic parameters, enzymes were mixed with varying substrate concentrations (0.5-200

167 μM). The FRET peptides were followed at ʎex 320 nm and ʎem 420 nm (excitation and emission

168 wavelengths for Abz) in a spectrofluorometer Shimadzu RF-1501 (Shimadzu Corporation, Kyoto, Japan),

169 previously calibrated with standard solutions of Abz hydrolyzed. KLK1 (5 nM) was assayed in 50 mM Tris-

170 HCl buffer pH 9.0 containing 1 mM of EDTA, using the substrate Abz-KLRSSKQ-EDDnp (Km 1.5 μM); KLK2

171 and KLK3 (5 nM) were assayed in 50 mM Tris-HCl buffer containing 1 M sodium citrate final pH 7.5

172 (adjusted with 2N NaOH after citrate addition), using the substrate Abz-KLRSSKQ-EDDnp (Km 5.9 μM

173 and 1.2 μM respectively); KLK5 (5 nM) and KLK6 (5 nM) were assayed in 50 mM Tris-HCl buffer pH 7.5

174 containing 1 mM of EDTA, using the substrate Abz-KLRSSKQ-EDDnp (Km 0.69 and 4.0, respectively); KLK7

175 (10 nM) was assayed in 50 mM Tris-HCl buffer containing 1 mM of EDTA, pH 7.5 using the substrate Abz-

176 KLFSSKQ-EDDnp (Km 1.8 uM). As an internal control of our inhibition study chymotrypsin (10 nM) was

177 assayed in 50 mM Tris-HCl buffer pH 8.0 containing 5 mM $CaCl_2$, using the substrate Abz-KLFSSKQ-

178 EDDnp (Km 4.0 μM) and trypsin (10 nM) was assayed in 50 mM Tris-HCl buffer pH 8.0 containing 5 mM

179 $CaCl_2$, using the substrate Abz-KLRSSKQ-EDDnp (Km 1.9 μM). To determine rmSpink3 inhibition constant,

180 assays were performed at 37°C at substrates concentration of 5 μM. The enzymes were pre-incubated in

181 the assay buffer with different concentrations of rmSpink3-$His_6$ for 3 min at room temperature before

182 substrate addition. Enzyme concentrations for initial rate determinations were selected such that less

183 than 5% of the added substrate would be hydrolyzed during the time course of data collection.

184 Enzymatic velocities in the presence or absence of rmSpink3-$His_6$ were calculated from the increments

185 of fluorescence in the time-course of the reactions and the values were expressed as the percentage of

186 the residual activity. The inhibitory domain of Kazal-type serine proteinase inhibitors is a typical

187 cysteine-rich motif, similar to the bovine pancreatic secretory trypsin inhibitor (PSTI), which prevents

188 protease activity in a substrate-like manner and binds tightly to the active site of the enzyme [35]. Thus,

189 it was assumed that rmSpink3 was a competitive tightly-binding inhibitor and the inhibition constants

190 (Ki) were obtained according the equation below [36, 37].

191

192 ***<u>Equation 1</u>***

193

$$[I]_t/((1 - v_i/v_0)) = \mathrm{K_i}\,(1 + S/K_m)\ v_i/v_0 + [E]_t$$

194

195 In the equation, *S* represents substrate concentration, *Km* the Michaelis-Menten constant, *[E*]

196 represents the enzyme concentration, *v0* the velocity of substrate hydrolysis at time zero and *vi* the

197 velocity at each inhibitor concentration. The reactions were performed at different inhibitor

198 concentrations [I] of and the Ki values were obtained by linear regression.

199

200 *2.4- Mass spectrometry analysis*

201 Trypsin (100 pmol) and rmSpink3-His$_6$ (1 nmol) were incubated in water (10 µL) for 30 minutes and the

202 released peptides were directly analyzed by MALDI-TOF/MS. Trypsin kept at least 20% its activity on Z-

203 FR-MCA in water. In five times higher concentration of trypsin we got the same result. Aliquots of

204 peptide mix (2µl) incubated with sinapinic acid (10 mg/ml) matrix solution (1:1) were spotted onto a

205 stainless steel MALDI target plate and dried at room temperature before analysis. Mass spectra were

206 obtained with a Bruker Daltonics Microflex LT instrument operating in linear, positive ion mode

207 previously calibrate with serum bovine albumin, proteinase A and trypsinogen. For analysis of protein

208 interactions, mass spectra were acquired utilizing the following instrument parameter: pulsed ion

209 extraction delay of 250 ns, ion source voltage one, 20 kV, ion source voltage two, 18.25 kV, and ion

210 source lens voltage 7.00 kV. For each sample, mass spectra were acquired by accumulating 50 laser

211 shots at 90% laser power in the m/z range of 18-40 kDa.

212

213 ***3- Results***

214 We have previously reported the construction of a recombinant vector encoding the fusion protein
215 GST-Spink3 [12] that was active as a trypsin inhibitor after removing the GST portion. However, protein
216 yields were not sufficient to allow further characterization of this molecule. In this study, we constructed
217 a new expression vector encoding recombinant C-terminal $His_6$ tagged Spink3 (rmSpink3-$His_6$) that was
218 synthesized as a soluble protein in *E. coli* (Figure 1, lane 2) and purified to apparent homogeneity by $Ni^{+2}$
219 -affinity chromatography (Figure 1, lane 3) with good yield (6 mg/L of culture). Its identity was confirmed
220 by Western blot with anti-hSPINK1 (the human orthologue protein) and by anti-His antibodies (Figure 1,
221 lanes 4 and 5, respectively). The activity of the recombinant protein was first assayed on trypsin showing
222 the typical competitive behavior described for Kazal type inhibitors (data not shown). Thereafter, the
223 effect of rmSpink3-$His_6$ on the activity of several recombinant KLKs was tested (Figure 2). Calculation of
224 the $IC_{50}$ from the inhibition plots showed that rmSpink3 was found to be a potent compound against
225 KLK2 and KLK5 ($IC_{50}$ = 4 and 4.1 µM, respectively), followed by KLK6 and KLK3 ($IC_{50}$ = 4.25 and 4.75 µM,
226 respectively). KLK7 $IC_{50}$ was not calculated since inhibition assay was performed with a different enzyme
227 concentration. These inhibitory concentrations are lower than those reported for natural isocoumarins
228 [38]. When the reaction was performed in the presence of high affinity substrates, substrate addition
229 reversed the inhibition caused by rmSpink3 or the inhibitor did not affect enzyme activity at all (for
230 example, KLK3 was not inhibited when Abz-KLFSSKQ-EDDnp was used as substrate; Km = 0.04 µM).
231 Although KLK3 is considered a chymotrypsin-like human kallikrein due to its high preference for Phe or
232 Tyr, Andrade et al [39] reported that Abz-KLRSSKQ-EDDnp is cleaved at R-S bond in presence of sodium
233 citrate with a Km value 25 times higher than the hydrolysis of Abz-KLFSSKQ-EDDnp. The evaluation of
234 inhibitors is more convenient using substrates with high Km and high kcat that was the case for the
235 hydrolysis of Abz-KLRSSKQ-EDDnp by KLK3 [39].

236 $IC_{50}$ values can vary with substrate concentration for competitive inhibitors such as Spink3. Moreover, in
237 a tight-binding inhibition model, formation of the EI complex (enzyme-inhibitor) changes the
238 concentration of free inhibitor during the reaction depending on the EI stoichiometry rather than the
239 absolute inhibitor concentration [37, 40]. Therefore, the EI dissociation constant (Ki) calculated using the
240 equation for tight-binding inhibitors as reported for other Kazal-type molecules [41], represents a better
241 way to evaluate the effect of rmSpink3 as protease inhibitor (Table 1). Inhibition assays using FRET
242 substrates indicated the strongest interaction between KLK5 and rmSpink3 (Ki = 0.26±0.03 µM) followed
243 by KLK2 (Ki = 0.63±0.03 µM). Slightly weaker inhibition was observed for KLK3 (Ki = 1.53±0.12 µM) and
244 to a lesser extent for KLK6 and KLK7 (Ki = 3.02±0.33 and 3.26±0.46 µM). Although KLK1 is a trypsin like
245 enzyme, its isoeletric point is the lowest among all kallikreins evaluated (Table I, inset). This fact might
246 explain the weak inhibition (Ki = 10.71±0.76 µM).
247 The rmSPIN3 Kis observed correspond well to those reported for Kazal type inhibitors ($10^{-7}$–$10^{-13}$ M)
248 [16]. The specificity of rmSpink3 differs from that previously reported for human SPINK6 [41] on
249 different KLKs. However, similarly to SPINK6, the target proteases of rmSpink3 have trypsin-like activity
250 most likely determined by Arg48, which is the predicted P1 residue of the inhibitor, as revealed by
251 sequence comparison with other Kazal-family protease inhibitors (Figure 3).
252 As described for canonical inhibitors, the standard EI mechanism implies that inhibitors are peculiar
253 protease substrates which contain the reactive site P1-P1' peptide bond located in the most exposed
254 region of the protease-binding loop [42]. Kazal-type molecules may or may not be cleaved during
255 inhibition [43]. As shown by the mass spectrometry analysis of rmSpink3 incubated overnight with or
256 without trypsin, the inhibitor molecule was not hydrolyzed by the enzyme (Figure 4).
257 In vivo, the role of mouse Spink3 as a serine protease inhibitor is not clear. Similarly to the human
258 orthologue hSPINK1, Spink3 activity is thought to participate in the prevention of trypsin-catalyzed
259 premature activation of zymogens within the pancreas [9]. Besides of pancreatic trypsin, their target

260 enzymes in other tissues are unknown. To explore the potential role of mSpink3 as an inhibitor of KLKs
261 activity, we examined the expression pattern of mSpink3, hSPINK1 and the KLKs used in this study using
262 an in silico approach, combining the information obtained from 4 databases (Gene Expression Atlas
263 EMBL-EBI, Unigene NCBI, Ensembl and MGI) (http://www.ebi.ac.uk; http://www.ncbi.nlm.nih.gov;
264 http://www.Ensembl.org; http://www.informatics.jax.org/). Based on this analysis, Spink3 is expressed
265 in many mouse tissues where KLKs are also expressed (Figure 5). In both mouse and human,
266 mSpink3/hSPINK1 are co-expressed with mKLK1/KLK1 in prostate, pancreas and uterus, suggesting that
267 in vivo KLK1 may be a target protease for Spink3. However, recombinant KLK1 showed the slightest
268 affinity for rmSpink3 *in vitro*. hSPINK1 is also co-expressed with KLK3 and KLK2 in human prostate, and
269 with KLK5 in uterus. As KLK2, KLK3 and KLK5 showed the highest affinities for rmSpink3-$His_6$ *in vitro*
270 (Table I), based on the structural and functional homology between hSPINK1 and hSpink3, it can be
271 speculated that hSPINK1 may contribute to the regulation of these KLKs in vivo.
272
273 ***4- Discussion***
274 Kazal-type serine protease inhibitors are typically small proteins (40 to 80 amino acids long), with a
275 conserved domain architecture including six cysteine residues that form three intra-domain disulfide
276 bridges in a characteristic pattern (CysI-CysV, CysII-CysIV and CysIII-CysVI). These bridges contribute to a
277 tight three-dimensional conformation which consists of one α-helix surrounded by a three stranded
278 β-sheet and loops of peptide segments [42]. The recombinant expression of these proteins in *E. coli*
279 usually represents an important challenge due to the presence of disulfide bonds, which may affect
280 protein solubility inside the cell and lead to misfolding and subsequent aggregation. We have been
281 working on the expression of recombinant SPINK3 motivated by the different roles that this protein has
282 been attributed, making it a multifunctional protein [7, 12]. In this context, the possibility that the
283 protein from mouse origin might be a natural molecule capable to inhibit human enzymes with

284 pharmaceutical importance was a challenge. This work shows that mouse Spink3, a member of typical
285 Kazal-type inhibitors, was expressed as a soluble protein in *E. coli* with good yield, and the recombinant
286 protein rmSpink3-$His_6$ proved to be a potent inhibitor of human KLK5, KLK2 and KLK3.
287 KLKs are interesting targets for the control of many human diseases [17]. They are secreted into various
288 biological fluids and increased levels of expression are unquestionable biomarkers, representing a target
289 point for the pharmaceutical industry.
290 The inhibition of the prostate-specific antigen PSA (KLK3) has been extensively studied. It was initially
291 found that 2-azetidinones inhibits active KLK3 [44]. Thereafter, structural modifications based on the
292 Ser-Ser-Lys-Leu-Gln peptide present in semenogelin II, which is a natural specific substrate for KLK3,
293 served as a template to obtain more potent inhibitors with Ki ranging 65 nm to 6.51 µM. Although they
294 had only a marginal effect in reducing development of cancer, labeled KLK3 inhibitors could find
295 application in diagnosis [17]. It was recently found that natural isocoumarins inhibit KLK5 (the KLK
296 involved in peeling skin syndrome) although their inhibition constants are in the micromolar range [17].
297 Recombinant mSpink3 inhibits KLK5 and KLK3 with Ki= 0.26 ± 0.03 and 1.53 ± 0.12 µM, respectively, thus
298 it can be considered as a powerful inhibitor of these KLKs.
299 Besides to synthetic molecules, natural peptides are also interesting candidates to control important
300 KLKs [23]. The activity of Kazal-type inhibitors toward KLKs has already been documented in vivo and *in*
301 *vitro*. Recently, SPINK6 was isolated from human skin [45] and has been described as a potent inhibitor
302 of KLK12 and KLK13 [41]. However, there is no evidence of the physiological role of these two enzymes,
303 with the exception that KLK12 expression is diminished in breast cancer [46]. On the other hand,
304 SPINK9/LEKTI-2 inhibits KLK5, considered as one of the most important KLKs in skin desquamation with a
305 Ki value similar to that observed in this work for rmSpink3 with the same enzyme [27]. The murine
306 orthologue of SPINK9, Spink12, inhibits trypsin like activity from primary murine keratinocytes, however
307 the authors of this work do not attribute this activity to KLKs [47]. Additionally, fragments of the

308 multidomain SPINK5/LEKTI-1 can efficiently and specifically inhibit the epidermal kallikreins KLK5, KLK7,

309 and KLK14 [48]. With an experimental approach similar to ours, human plasma kallikrein (KLKB1) was

310 also inhibited *in vitro* by a non-typical Kazal-type inhibitor, AdKI (2.9 kDa polypeptide) isolated from the

311 Cuban mollusk *Aplysia dactylomela* being also identified and characterized [49]. Regarding the inhibitory

312 activity of SPINK1 on kallikreins, it has been reported that PSTI from human gastric mucose was not

313 effective against pancreatic porcine kallikrein [8], however this kallikrein is referred by MEROPS

314 database as KLKB1 [50], which was not assayed by us.

315 Compared to this previous reports for the inhibition of KLKs by Kazal type inhibitors, our results are Kis

316 higher than the ones reported for SPINK6 [41] or SPINK5/LEKTI fragments [51], meaning less efficient

317 inhibition. However, our inhibition constant values correspond well to those reported for Kazal type

318 inhibitors ($10^{-7}$–$10^{-13}$ M) [16].

319 The control of excessive activity of KLKs is a result of the balance between these enzymes and their

320 specific inhibitors. Thus, it is hypothesized that in diseases in which the expression of KLKs is increased, a

321 compensatory raise in the expression of inhibitors should occur. In this sense, mSpink3 is believed to

322 have essential roles in the maintenance of integrity and regeneration of acinar cells, since *Spink3-/-* mice

323 showed enhanced trypsin activity [10] and autophagic degeneration of acinar cells but no signs of

324 apoptosis [3]. The human orthologue SPINK1 is thought to function in the prevention of trypsin-

325 catalyzed premature activation of zymogens within the pancreas and the pancreatic duct [9] and

326 mutations in this gene are associated with hereditary pancreatitis [52]. Its expression is augmented in

327 breast cancer [53] and because of that it has been recently proposed as an *outlier marker* (i.e., a protein

328 with enhanced expression in a subset of cases) in prostate cancer [54, 55]. This increased expression

329 might probably be a mechanism to control the increased activity of KLK3 [56]. Spink3 was also found on

330 the mouse sperm surface bound to an unknown ligand [12, 15] from where it detaches within the

331 female duct and was reported to inhibit a trypsin-like activity from uterine fluid [15].

332 Regardless of the evidences cited above, the target proteinases for mSpink3 and hSPINK1 in organs
333 other than pancreas are unknown. Based on the analysis of in silico data one can speculate that mSpink3
334 might be a potential inhibitor of mKLK1 since both proteins are expressed simultaneously in prostate,
335 pancreas and uterus. This co-expression pattern is consistent with the human orthologues, meaning that
336 there is some functional conservation. Additionally, considering that rmSpink3-$His_6$ was a potent
337 inhibitor of KLK5 *in vitro* (Table 1) it can be speculated that the human orthologue SPINK1 would inhibit
338 KLK5 in vivo. This is supported by the fact that these proteins are co-expressed in the same tissues (for
339 example, stomach and uterus). Similar extrapolation might be done for SPINK1 and KLK2/KLK3 which are
340 co-expressed in prostate.
341 In conclusion, our efforts to find novel KLK inhibitors resulted in the efficient production of recombinant
342 Spink3 from mouse seminal vesicle which showed high inhibitory activity on several tissue human
343 kallikreins. New target KLKs for mSpink3 and hSPINK1 are proposed based on in silico analysis of
344 expression; however, this observation needs experimental validation using the right partners in further
345 studies.
346
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351
352
353 ***6- Glossary***
354 KLK: Kallikrein
355 SPINK: Serine protease inhibitor Kazal-type

356 FRET: Fluorescence resonance energy transfer

357 GST: glutathione S-transferase

358 PSA: Prostate specific antigen

359 KLKB1: Human plasma kallikrein

360 kDa: kilo Dalton

361 His: Histidine

362 Ki: Inhibition constant

363 Km: Michaelis constant

364 PSTI: Pancreatic secretory trypsin inhibitor

365 MALDI: Matrix-assisted laser desorption/ionization

366 EI: Enzyme-inhibitor complex

367

368 Legends to the figures

369

370 **Figure 1: Recombinant expression and purification of rmSpink3.** Electrophoretic profile by SDS-PAGE of

371 the soluble fractions of *E. coli* cells harboring pET-24b (+)-*spink3-His*$_6$ after 16 h in absence (-IPTG, lane

372 1) or presence (+ IPTG, lane 2) of 0.1 mM IPTG**.** rmSpink3-His$_6$ was purified by HiTrap affinity

373 chromatography ($Ni^{2+}$, lane 3). Protein samples were stained with Coomasie brilliant blue G250 (lanes 1-

374 3). Purified rmSpink3-His$_6$ was identified by western blotting with anti-SPINK1 (α-SPINK1, lane 4) and

375 anti-His (α-HIS, lane 5) antibodies. The positions of molecular mass markers (kDa) are indicated on the

376 left. A representative gel of three independent experiments is shown.

377

378 **Figure 2: Inhibition of KLKs by rmSpink3**. Recombinant human kallikrein related peptidases (KLKs) were

379 synthesized from an insect cell/baculovirus expression system as described in M&M. The enzymes (5 nM

380 except of KLK7, tryspin and chymotrypsin that were 10 nM) were pre-incubated with increasing

381 concentrations of rmSpink3 for 3 min at room temperature. After incubation, the fluorescence

382 resonance energy transfer (FRET) peptides Abz-KL**R**SSKQ-EDDnp or Abz-KL**F**SSKQ-EDDnp were added at 5

383 µM concentration as indicated. The reaction progress was monitored in the linear range of the enzyme

384 kinetic curve and the residual activity was plotted against rmSpink3 concentration.

385

386 **Figure 3: Mouse SPINK family.** ClustalW2 sequence alignment of mouse SPINK family members

387 including human SPINK1 that was italicized. Each domain of mSPINK5 has been aligned separately. P1

388 and P1' sites are framed and Cys residues are shaded. Amino acid sequences of SPINK family inhibitors

389 were obtained from the Uniprot databank (http://www.uniprot.org/). Only the portion corresponding to

390 the mature form of the proteins is indicated as predicted by SignalP software

391 (http://www.cbs.dtu.dk/services/ SignalP-2.0/).

392

393 **Figure 4: Kazal-type mechanism determination using MALDITOF mass spectrometry**. Purified rmSpink3
394 (1 nmol) was incubated with or without Trypsin (100 pmol) in water for 30 minutes. The prepared
395 samples were directly analyzed by MALDI-TOF/MS.

396

397 **Figure 5: Tissue distribution of Spink3, SPINK1 and KLKs expression in mouse and human**. Expression
398 data either at the mRNA or protein level were obtained by combining the information obtained from 4
399 databases (Gene Expression Atlas EMBL-EBI, Unigene NCBI, Ensembl and MGI) (http://www.ebi.ac.uk;
400 http://www.ncbi.nlm.nih.gov; http://www.Ensembl.org; http://www.informatics.jax.org/). Only the
401 assayed human kallikreins (KLKs) and their mouse orthologues (mKLKs) are included. Bold KLKs indicate
402 KLKs inhibited by rmSpink3.

403

404 **Table 1: Kinetic parameters of KLKs interaction with rmSpink3-His$_6$**
405 Various recombinant KLKs were incubated with increasing concentrations of rmSpink3-His$_6$ and the
406 residual activity was determined using the corresponding FRET substrate as indicated in Figure 2. The
407 inactivation constants (Ki) were obtained according the Henderson equation for tight-binding inhibitors
408 [36] where [I]t/(1-vi/v0 ) =Ki (1+S/Km) vi/v0 +[E]t .Data are presented in µM±SEM between at least 3
409 replicates. Inset= Calculated isolectric points for recombinant KLKs.

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413 ***6- References***


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