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Kallikrein Protease Activated Receptor (PAR) Axis: An Attractive Target for Drug Development

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INTRODUCTION

All living prokaryotic and eukaryotic organisms, from bacteria to mammals, as well as viruses, express proteolytic enzymes with similar or distinct functions.[1−4] Apart from their well appreciated roles in protein catabolism, generating active polypeptides from protein precursors and functioning as digestive enzymes, several proteinases are now known to play a hormone-like role by triggering signal transduction pathways in target cells; for example, it is well-known that thrombin, a classical serine protease, circulates in the blood to reach its target tissues. The serine proteinase family currently numbers 176 members, which represent about 30% of the known proteolytic enzymes[5,6] and most are secreted enzymes.[7] Chromosomes 19 and 16 contain the two largest clusters of serine proteinases: the kallikrein family and the tryptase family, respectively, both belonging to the family S1, clan SA (currently known as clan PA) of serine proteinases along with chymotrypsin, trypsin, and elastase.[6−8] Kallikrein 1 (KLK1), or the tissue kallikrein, has long been defined as glandular kallikrein to distinguish it from the later discovered plasma kallikrein (KLKB1). KLKB1 and KLK1 are serine endopeptidases that cleave high and low molecular mass kininogen (HMWK and LMWK, respectively), generating bradykinin and Lys- bradykinin, respectively. KLKB1, which is part of the plasma contact activation system, is a complex multidomain protein and structurally related to coagulation factor XI, whereas KLK1 displays a simpler structure and related to that of trypsin. Mason et al.[9] demonstrated that the mouse and the rat genomes carry several genes that are closely related to KLK1, but for a long time, only three glandular kallikreins were identified in humans: KLK1,[10] KLK2,[11] and KLK3,[12] also known as prostate-specific antigen (PSA). At around the turn of the millennium, three research groups independently discovered that the human kallikrein locus on the long arm of chromosome 19 carries a number of genes encoding serine endopeptidases that are related to KLK1.[13−15]

Human kallikreins (hK) are a multigene family of 15 secreted serine type proteases situated 7.5 Mb from the telomeres of the long arm of chromosome 19, in the cytogenic region q13.3−4.[14,16−18] The locus spans 265 kb and carries 15 functional genes and at least 1 pseudogene. According to the recent nomenclature, kallikreins are now indicated as kallikrein-related peptidases, with the exception of KLK1, for which the older nomenclature has been retained.[19] Gene and protein numbers are assigned in sequence starting from the centromere to the telomere (Table 1).

Table 1. Kallikrein-Related Peptidases Family: Gene and Protein Nomenclature[a]

gene	other gene symbols	protein	other protein names
KLK1		KLK1	pancreatic/renal/urinary kallikrein
KLK2		KLK2	human glandular kallikrein
KLK3		KLK3	prostate-specific antigen (PSA)
KLK4	*PRSS17, KLK-L1*	KLK4	prostase, KLK-L1 protein, EMSP1
KLK5	*KLK-L2*	KLK5	KLK-L2 protein, SCTE
KLK6	*PRSS9*	KLK6	zyme, protease m, neurosin
KLK7	*PRSS6*	KLK7	SCCE
KLK8	*PRSS19, TADG14*	KLK8	neuropsin, ovasin
KLK9	*KLK-L3*	KLK9	KLK-L3 protein
KLK10	*PRSSL1*	KLK10	NES1
KLK11	*PRSS20*	KLK11	TLSP, hippostasin
KLK12	*KLK-L5*	KLK12	KLK-L5 protein
KLK13	*KLK-L4*	KLK13	KLK-L4 protein
KLK14	*KLK-L6*	KLK14	KLK-l6 protein
KLK15	*HSRNASPH*	KLK15	prostin

[a]Adapted from ref 17.

Within the human family, homology reaches the level of 30−50% at the gene and protein level, with the exception of KLKs 1−3 that show more striking similarities when compared to each other (80%).[18−20] Even the mouse KLK and rat KLK gene families are composed of a large number of closely related members that possibly arose from gene duplication events.[9,21,22] Similarly, the number of the kallikrein isoforms may be even higher than these 15 members because of alternative splicing of kallikrein genes; a recent review has reported 82 different kallikrein gene transcript forms, including reference forms.[23] The members of mouse, rat, and human kallikreins have a high degree of amino acid identity but present different substrate specificity, particularly toward oligopeptides.[24−29] The protein products of the genes are characterized by a 16−34 residue amino terminal signal peptide that leads them to the endoplasmic reticulum for secretion. Thus, the signal peptide is cleaved off to yield the inactive zymogen form of the peptidase, from which the fully active enzyme is generated by removal of the propeptide by specific proteolysis. Kallikrein-related peptidases show a high degree of homology with trypsin; this similarity is not limited to

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Table 2. Human KLKs as Biomarkers of Prostate, Breast, and Ovarian Cancer[a]

cancer type	gene or protein[b]	clinical application	cancer type	gene or protein[b]	clinical application
prostate	KLK2	diagnosis, prognosis, and monitoring	breast	KLK3/PSA	diagnosis, favorable prognosis, resistance to therapy
prostate	KLK3/PSA	diagnosis, prognosis, and monitoring	breast	KLK5	unfavorable prognosis
prostate	KLK5	favorable prognosis	breast	KLK5	diagnosis,
prostate	KLK10	unfavorable prognosis	breast	KLK7	unfavorable prognosis[50]
prostate	KLK11	favorable prognosis	breast	KLK7 (full length variant)	favorable prognosis[51]
prostate	KLK11	diagnosis	breast	KLK9	favorable prognosis
prostate	KLK14	unfavorable prognosis	breast	KLK10	resistance to therapy
prostate	KLK15	unfavorable prognosis	breast	KLK10	predictive of invasiveness
breast	KLK14	unfavorable prognosis	breast	KLK13	favorable prognosis
breast	KLK14/KLK14	diagnosis[48]	breast	KLK15	favorable prognosis
ovarian	KLK4	unfavorable prognosis	ovarian	KLK9	favorable prognosis
ovarian	KLK4	resistance to therapy[49]	ovarian	KLK10	unfavorable prognosis, diagnosis, and monitoring
ovarian	KLK5	unfavorable prognosis	ovarian	KLK10	unfavorable prognosis, diagnosis[52]
ovarian	KLK5	unfavorable prognosis and diagnosis	ovarian	KLK11	favorable prognosis and diagnosis[53]
ovarian	KLK6/KLK6	unfavorable prognosis	ovarian	KLK11	unfavorable prognosis
ovarian	KLK6	diagnosis and monitoring	ovarian	KLK13	favorable prognosis
ovarian	KLK7/KLK7	unfavorable prognosis	ovarian	KLK14	favorable prognosis
ovarian	KLK8/KLK8	favorable prognosis	ovarian	KLK14	diagnosis
ovarian	KLK8	diagnosis and monitoring	ovarian	KLK15	unfavorable prognosis

[a]Adapted from refs 17 and 46. [b]*KLK* = kallikrein-related peptidase gene. KLK = kallikrein-related peptidase protein.

the primary structure, as they display similar catalytic mechanism. All but four kallikrein-related peptidases present an Asp residue homologous to that of trypsin, located at the base of the substrate-binding pocket, addressing the specificity toward positively charged Lys and Arg residues. A tryptic or Arg restricted specificity has been confirmed with KLK2, KLK4, KLK5, KLK6, KLK8, KLK10, KLK12, KLK13, and KLK15, while the activity is chymotrypsin-like for KLK7 and KLK9, which present at their S1 site Ser or Asn or Gly, or mixed-type for KLK3, KLK11, and KLK14.[28−32]

The natural substrates and the substrate specificities of the kallikreins are known only for a few of them. KLK1 is the best characterized kallikrein. It is a glycoprotein expressed most abundantly in pancreas, salivary gland, kidney, and urine, releases Lys-bradykinin by limited proteolysis from high and low molecular weight kininogens by cleavage at the Met379−Lys380 and Arg389−Ser390 bonds, and has both trypsin- and chymotrypsin-like activities.[33−38] KLK2 is present in the seminal plasma, hydrolyzes certain components of the semen coagulum, and cleaves substrates with restrictive trypsin-like specificity.[39] In contrast, KLK3/PSA has only chymotrypsin-like activity and is expressed in the glandular epithelial cells of the prostate gland. KLK3 rapidly hydrolyzes both semenogelin I and semenogelin II in vivo and synthetic substrates derived from these proteins and was reported to be strongly activated by Na_2SO_4 and by heparin.[40−42] KLK6 is possibly involved in myelin turnover and demyelination processes, modulation of ionotropic glutamate receptors, and activation of PAR2.[30] Recently crystal structures of KLK4, KLK5, KLK6, and KLK7 have been available. By combination of these data with enzyme kinetic studies and extended substrate specificity profiling, a better understanding of the non-prime-side substrate preferences of these peptidase has been obtained. The shape and polarity of the specificity pockets S1−S4 confirm the trypsin-like specificity for KLK4, KLK5, and KLK6 in contrast with KLK7, displaying a unique chymotrypsin-like specificity for Tyr, which is also preferred at P2.[43]

KALLIKREIN-RELATED PEPTIDASES AS HORMONES

As mentioned above, KLKs are expressed in several tissues as inactive preproforms that are processed to inactive proforms, secreted into the circulation and other major biological fluids, such as cerebrospinal fluid, vaginal fluid, nipple aspirate fluid, and tumor ascites fluid.[17] Mature peptidases are then obtained by specific proteolytic removal of their N-terminal propeptide either via autocatalytic activity or by another KLK or other endopeptidases. After secretion, KLKs can migrate through the bloodstream or other biological fluids to surrounding and/or distant cells to reach their targets, exerting their biological actions by means of proteolysis. Several processes, mainly represented by (auto)activation, (auto)degradation, and inhibition by serine proteinase inhibitors (SERPINs), have been evolutionarily produced in order to functionally control KLK activity and to eliminate any chance of unwanted or unlimited proteolysis. Mature KLK enzymes, for example, are amenable to inactivation by endogenous inhibitors such as kallistatin that inhibits KLK1, KLK7, and KLK14[44,45] and LEKTI (lymphoepithelial Kazal-type inhibitor), which is encoded by SPINK5 (serine protease inhibitor Kazal-type 5).[29] A remarkable feature of KLKs is that they share a wide range of expression in different tissues; groups of kallikreins, in fact, are coexpressed in several physiological and pathological settings. This observation has produced several hypotheses including a common biological role or cooperativity among the coexpressed peptidases. KLK coexpression is represented, for example, by KLKs 2, 4, 11, 15 in the prostate, KLKs 1, 4, 11, 13, 14 in the skin, KLKs 5, 6, 10, 13 in the breast, and KLKs 6, 9, 14 in CNS.[17,20]

KLK Expression in Cancer and Inflammation. Aberrant KLK expression patterns have been reported mainly in hormone dependent malignancies, such as those in breast, ovary, and prostate, and have been widely implicated as cancer biomarkers (Table 2).[17,20,46] Most of the evidence obtained so far points to dual roles of KLKs in cancer, as their function may depend on the tissues, tumor types, and cancer stages. Moreover, concentration and/or activity levels have to be taken in account, as also

Table 3. Human KLKs as Biomarkers of Other Types of Cancer (Testicular, Uterine, Cervical, Brain, Head and Neck SCC, Skin SCC, Melanoma, Colon, Pancreas, Leukemia)[a]

cancer type[b]	gene or protein[c]	expression and/or clinical application	cancer type[b]	gene or protein[c]	clinical application
testicular	KLK5	favorable prognosis	brain	KLK6	up-regulation
testicular	KLK10	favorable prognosis	brain	KLK7	unfavorable prognosis[59]
testicular	KLK13	diagnosis	uterine	KLK6	monitoring and prediction to therapy[60]
testicular	KLK14	favorable prognosis	uterine	KLK8/KLK8	up-regulation[61]
cervical	KLK7/KLK7	up-regulation	uterine	KLK10	diagnosis[62]
cervical	KLK8/KLK8	up-regulation	salivary gland	KLK6	down-regulation[63]
head and neck SCC	KLK10	up-regulation	salivary gland	KLK13	up-regulation[64]
non-small-cell lung AC	KLK5	up-regulation[54]	skin SCC	KLK8	up-regulation
non-small-cell lung AC	KLK7	down-regulation[54]	skin SCC, melanome	KLK6/KLK6	up-regulation[65]
non-small-cell lung AC	KLK8	favorable prognosis[55]	colon	KLK1	down-regulation
non-small-cell lung AC	KLK10/KLK11	up-regulation[56]	colon	KLK6	up-regulation
neuroendocrine C2 lung AC	KLK11	unfavorable prognosis	colon	KLK8	up-regulation
non-small-cell lung AC	KLK13/KLK14	up-regulation[57]	colon	KLK10	up-regulation
leukemia	KLK10	down-regulation	pancreas	KLK6	up-regulation
RCC	KLK6	unfavorable prognosis[58]	pancreas	KLK10	up-regulation

[a]Adapted from refs 17 and 46. [b]AC = adenocarcinoma;. SCC = squamous cell carcinoma. RCC = renal cell carcinoma. [c]*KLK* = kallikrein-related peptidase gene. KLK = kallikrein-related peptidase protein.

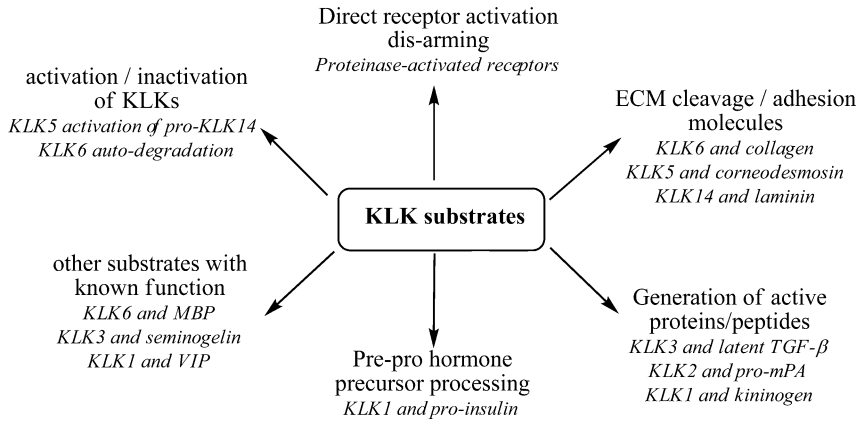

Figure 1. Potential substrates via which KLKs can regulate tissue function. Selected examples are given for each suggested substrate group. MBP stands for myelin basic protein, VIP for vasoactive intestinal peptide, TGF-β for tissue growth factor type β, and pro-uPA for the zymogen of urokinase plasminogen activator. For details about the potential KLK substrates, see refs 17 and 20.

described for other proteases.[7] As is known, prostate cancer cells produce a large amount of PSA/KLK3, and it is used extensively as a valuable biomarker for monitoring prostate cancer, detecting recurrence after local therapies, and to follow response to systemic therapies for metastatic disease. Accumulating evidence suggests that PSA may be more than just a biomarker and may play a role in the pathobiology of prostate cancer.[47] Other kallikreins such as KLK2, KLK4, KLK5, KLK6, KLK7, KLK8, KLK9, KLK10, KLK11, KLK13, and KLK15 were found to be candidate biomarkers for several endocrine-related malignancies.

In addition to hormone-related malignancies, KLK expression has been related to several other types of tumors, summarized in Table 3.

Up-regulation of KLK6 and KLK10 has been identified in uterine serous papillary tumors, an aggressive type of endometrial cancer. These two KLKs are overexpressed in pancreatic and colon cancer. KLK7 and KLK 8 have been related to cervical cancer, one of the most common gynecological tumor (Table 3), while KLK11 is overexpressed in a specific subgroup of neuroendocrine lung carcinomas. KLK5, KLK10, and KLK14 may possess clinical value for testicular cancer prognosis, and KLK13 may aid in diagnosis of this type of cancer (Table 3).

The biological roles of KLK1 in inflammation have been thoroughly studied.[66] It has been shown in vitro that some of KLKs can proteolytically activate hormones and growth factors, other proteinases, or extracellular matrix components possibly affecting tissue function (Figure 1).[17,20]

As mentioned above, KLKs can also induce cell proliferation, as keratinocytes;[65] furthermore, it has long been known that KLK1 has mitogenic effects by generating active kinin peptides from their kininogen precursor (Figure 1). The kinins can subsequently trigger the activation of the bradykinin B2 G-protein-coupled receptor.[67] Recent studies have shown that KLKs can regulate cell signaling by cleaving and triggering the activation of protease-activated receptors (PARs) (Figure 1).[30,68,69]

PARs 1−4 are members of the G-protein-coupled receptor superfamily. They have been implicated in a number of physiological and pathological signaling pathways in a variety of tissues.[70−72] Thus, these receptors are additional targets by

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Table 4. PAR Family[a]

	receptor			
	PAR1	PAR2	PAR3	PAR4
amino acid composition	425 aa (h)	397 aa (h)	374 aa (h)	385 aa (h)
tethered ligand sequence	SFLLR (h)	SLIGKV (h)	TFRGAP (h)	GYPGQV (h)
	SFFLR (m, r)	SLIGRL (m, r)	SFNGGP (m)	GYPGKV (h)
selective agonist peptide	TFLLR-NH_2	SLIGRL-NH_2	unknown	AYPGKF-NH_2
major activating proteinase	thrombin	trypsin tryptase	thrombin	thrombin trypsin
major disarming proteinase	trypsin cathepsin G	elastase, chymase	cathepsin G	unknown

[a]h stands for human, m for mouse, and r for rat receptors.

which KLKs, like hormones, can transmit their chemical messages in an endocrine, paracrine, or autocrine manner.

KALLIKREINS AS REGULATORS OF PROTEINASE-ACTIVATED RECEPTORS SIGNALING

Accumulating evidence suggests that KLKs are activators of PARs that comprise four G-protein-coupled receptors (PAR1, PAR2, PAR3, and PAR4). They are irreversibly activated by the action of proteinases mainly from serine proteinase class and with trypsin-like substrate specificity for cleavage following arginine or lysine residues.[73−75] Accumulating evidence suggests that the cleavage occurs within the amino terminal exodomain of the receptor following either an arginine or lysine residue, generating a new amino terminal. This unmasked tethered ligand binds intramolecularly, causing allosteric changes within the PAR, followed by receptor coupling to heterotrimeric G proteins and signal transduction. Importantly, proteinases may also cleave downstream of the activation site or within a PAR extracellular loop, leading to disarming of the receptor.[68] Synthetic peptides mimicking the tethered ligands can also activate PARs even in the absence of proteinases intervention.[73] These synthetic receptor-activating peptides are named PAR-activating peptides (PAR-APs). They have been proved to be key reagents for the study the pharmacology of the PARs and their role in pathobiology. Moreover, they have been employed as starting point for the design of novel receptor ligands in order to assess the key structural features needed for the interaction with the receptor. The obtained results in terms of both involvement in pathophysiological conditions and research of PARs agonists and antagonists have been extensively reviewed.[71,72,76−79]

The characteristics of PARs family are summarized in Table 4.

Stemming from studies with both PAR-activating peptides and receptor antagonists, as well as PAR-null mice, it has become evident that the PARs and therefore their activating/inactivating proteinases play important roles in a number of pathophysiological processes, such as cardiovascular responses,[80−83] neuronal cell survival,[71,84,85] platelet aggregation, inflammation, and epidermal function.[86−88] Moreover, PAR receptors are involved in mediating disease-associated cellular changes including those necessary for cancer progression such as dysregulated cell proliferation and migration.[75,89−92]

KLKs and PARs in Cancer and Inflammation. Several studies have been performed confirming that KLK signaling via PARs is associated with both inflammation and cancer. Much of the data supporting a role for KLKs in regulating cell function have come from studying the effects of KLKs on PAR expressing cell lines in vitro. KLK4, highly expressed in normal prostate, has been associated with prostate cancer progression, and this effect has been correlated to PARs.[93] Confocal microscopy analyses indicated that KLK4 initiates loss of PAR2 from the cell surface and receptor internalization. Immunohistochemical analysis indicated the coexpression of KLK4 and PAR2 in primary prostate cancer and bone metastases. By use of PAR1 knockout mouse lung fibroblast cell line transfected with PAR1 (KOLF-PAR1) or PAR2 (KOLF-PAR2), it was demonstrated that KLK4 activates both PAR1 and PAR2 whereas KLK2 activates PAR2.[94] KLK4 interaction with PAR1 and the subsequent initiation of ERK1/2 signaling can play a major role in tumor−stroma interactions in androgen-independent prostate cancer.[95] A similar paracrine function has also been suggested for KLK6 strongly expressed in keratinocytes and stromal cells located adjacent to benign nevi, primary melanomas, and cutaneous metastatic lesions.[96] Finally, KLK4 and KLK14 signaling via PAR1 and PAR2, respectively, are involved in colon tumorigenesis.[97,98]

KLKs and PARs in Skin Pathology. Other possible pathophysiological roles for local kallikreins may be found in the skin, where PARs and kallikreins are coexpressed either in normal or in pathological settings such as psoriasis. KLKs 5, 7, and 14 have been isolated in their active forms from the outermost layers of the stratum corneum.[99,100] KLK8 is another human KLK, which is abundantly present in the stratum corneum.[101] A KLK cascade involving KLKs 5 and 7 has been identified and may effect skin desquamation via the degradation of intercellular (corneo)desmosomal adhesion molecules.[99,102] By use of immunofluorescence analysis and functional activation by measurements of changes in intracellular calcium levels, it was demonstrated that KLK5 and KLK14, but not KLK7 or KLK8, induced PAR2 signaling.[103] KLK5, in particular, can play a major role in pathobiology of the Netherton syndrome (NS), a rare genetic skin disease with a profound skin barrier defect and severe allergic manifestations.[104] Briot et al. have demonstrated that KLK5, via PAR2 signaling, increases expression of proinflammatory cytokines and chemokines such as thymic stromal lymphopoietin (TSLP), tumor necrosis factor α, IL-8, and intercellular adhesion molecule 1 (ICAM-1) in human primary keratinocytes.[105] Moreover, a novel signaling mechanism of KLK1 in promoting skin cell migration and wound healing in keratinocytes has been described. KLK1's stimulatory effect was inhibited by small interfering RNA for PAR1 and by PAR1 antagonist, demonstrating the involvement of the PAR1−PKC−Src−MMP pathway.[106]

KLKs and PARs in CNS Pathology. A number of KLKs are expressed in the central nervous system. Among them, KLK6 is the most studied, and its involvement in several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis has been documented.[107,108] In particular, it has been suggested that KLK1 and KLK6 are directly implicated in neurodegeneration and could be useful tools to follow multiple sclerosis progression.[109] Starting from the consideration that PARs are highly expressed in both the central

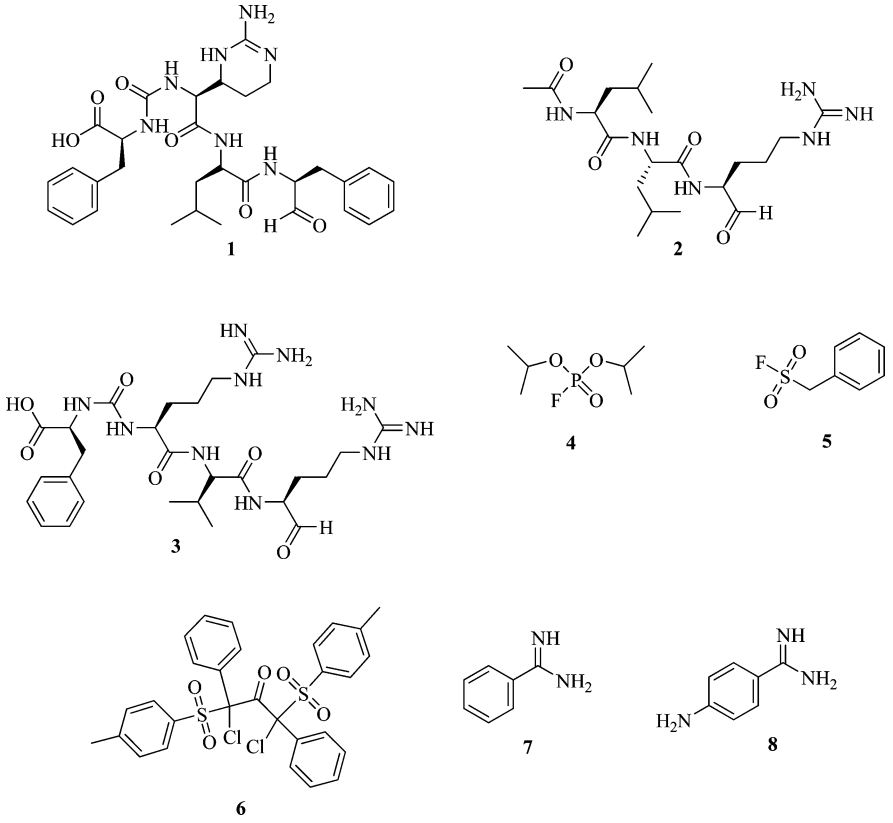

Figure 2. Low specificity small peptides and small molecule KLK inhibitors.

and peripheral nervous system where PAR1 and PAR2 are primarily responsible for triggering inflammation, it is hypothesized that a pathological role of KLKs/PARs axis is also in this setting. Supporting data for this hypothesis are that PAR2, whose activity can be regulated by KLKs, has been implicated in a murine experimental autoimmune encephalitis model of multiple sclerosis.[110] A recent study demonstrated that KLK6, but not KLK1, signals through PARs. KLK6 evoked intracellular Ca^{2+} flux was mediated by PAR1 in neurons and both PAR1 and PAR2 in astrocytes.[111] The KLK6-mediated PAR1 activation also participates in promoting lymphocyte survival. Insufficient lymphocyte apoptosis is a well-known participant in autoimmune and lymphoproliferative disease but in excess can promote immunodeficiency. Scarisbrick et al. demonstrate for the first time that KLK6 is able to promote survival of murine splenocytes as well as the human leukemic Jurkat T cell line, suggesting a novel molecular mechanism regulating lymphocyte survival that could be important for a range of immunological responses that depend on apoptosis for immune clearance and maintenance of homeostasis.[112]

KALLIKREINS AND PARS AS THERAPEUTIC TARGETS

Given the wide range of pathophysiological conditions in which PARs are involved, it is expected that their activation or inhibition would be critical for many pathological conditions. This consideration renders the kallikrein−PAR axis an attractive target for drug development. Research on kallikrein inhibitors has led to compounds with insufficient enzyme specificity. A great abundance of serine proteinases are able to activate PAR receptors, rendering kallikreins attractive diagnostic and therapeutic targets, only in particular pathological conditions such as prostate cancer and skin diseases. Research on PARs antagonists has produced interesting results particularly regarding design and synthesis of PAR1 antagonists and their use in cardiovascular diseases. PAR1 antagonists are expected to be safer than currently available antiplatelet agents, since they do not influence thrombin induced cleavage of fibrinogen, which is the first step in coagulation. Additionally, experimental and clinical data show the roles of PARs regulating several inflammatory responses, as well as tumor growth and metastasis.

KALLIKREIN INHIBITORS

Several natural inhibitors, such as the small peptides chymostatin (**1**), leupeptin (**2**), antipain (**3**), the polypeptides soybean trypsin inhibitor (SBTI) and aprotinin and/or synthetic small molecule inhibitors, such as diisopropyl fluorophosphate (DIFP, **4**), phenylmethylsulfonyl fluoride (PMSF, **5**), and tosylphenyl chloromethyl ketone (TPCK, **6**) have been used in order to gain insight on the primary substrate specificity of the respective kallikrein-related peptidase, allowing also a more complete characterization of the enzymes (Figure 2).[113]

Despite the usually low specificity of these inhibitors, the data obtained allowed the design of more specific compounds. Aldehydic peptides **1**, **2**, and **3**, in which the C terminal residue is arginal or phenylalaninal, are produced by microorganisms as protease activity attenuators or inhibitors that target host proteases.[114,115] These peptides act by forming a hemiacetal adduct between their aldehyde group and Ser and/or Cys nucleophiles, affording a transition-state-like inhibiting protease complex, which is reversible. Peptides **2** and **3**, characterized by the presence of an Arg residue at P1 position, are more specific for triptic KLKs, while **1**, presenting a leucylphenylalaninal moiety, is directed against chymotryptic proteases. Other small

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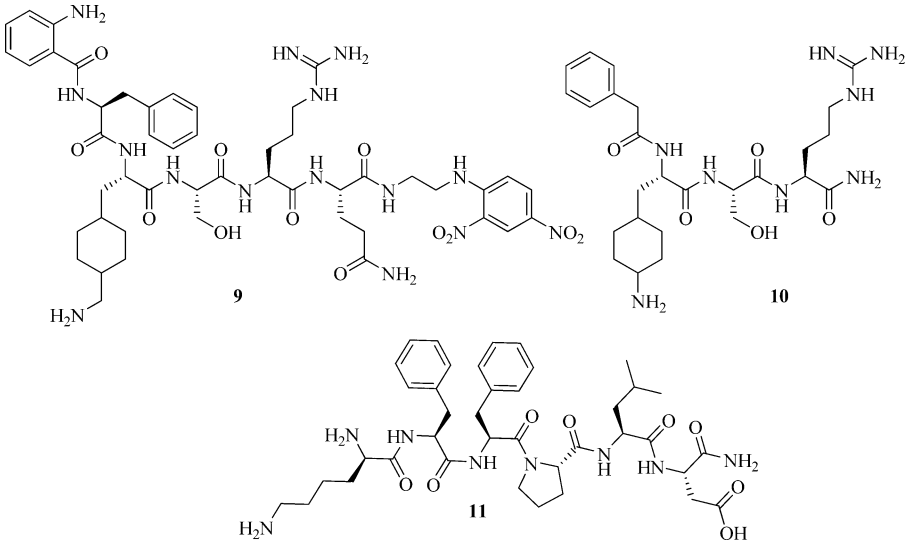

Figure 3. Peptide and peptide-mimetic KLK1 inhibitors obtained by drug design approach.

molecules, mainly employed for purification and/or characterization purposes, since their low specificity and/or high toxicity does not allow a therapeutic development, are (i) benzamidines, (ii) sulfonyl fluorides and diisopropyl fluorophosphate, and (iii) chloromethyl ketones. Benzamidines (such as benzamidine (**7**) and *p*-aminobenzamidine (**8**)) are tryptic inhibitors, since their aromatic rings fit the S1 pocket and their amidino moieties interact with the negatively charged Asp189 (Figure 2). Because of the absence of other interactions with the enzymes, these inhibitors show inhibition constants ranging from high micromolar to millimolar values. Nevertheless, these molecules have been very useful in crystallographic studies. In fact more than 100 crystal structures of **7** or **8** complexed with several proteases have been reported. Compounds **4** and **5** are irreversible and toxic protease inhibitors that react with serine and cysteine residues inhibiting serine protease but also esterase enzymes. Chloromethyl ketones are also irreversible inhibitors that covalently link the enzymes but, similar to aldehydic derivatives, allow the design of more specific compounds. Tosyllysyl chloromethyl ketone (TLCK, structure not disclosed), for example, was an efficient inhibitor for KLKs 1, 4, and 8, while KLK7 was successfully crystallized with Ala-Ala-Phe-CMK and succinyl-Ala-Ala-Pro-Phe-CMK (structures not disclosed).[116−118]

Designing KLK Inhibitors. Designing KLKs inhibitors has been very challenging because of the sequence homology within the 14 members of the family that usually does not permit highly selective molecules that could interact with just one or a few members of the family.

The increasing amount of information regarding the three-dimensional structures of many KLKs in conjunction with the advance in synthetic methodologies and high-throughput screening assays has stimulated several research groups to prepare small molecules able to inhibit several components of the KLK family. The interest has been focused particularly on those KLKs that appear as attractive diagnostic and therapeutic targets, such as the members of the family that are involved in prostate cancer and skin diseases.[119]

Peptide and Peptide-Mimetic KLK Inhibitors. In 2001, Juliano et al. presented the first description of substrates or inhibitors for KLK1 using internally quenched fluorescent peptides Abz-F-X-S-R-Q-EDDnp and Abz-G-F-S-P-F-X-S-S-R-P-Q-EDDnp [where Abz is *o*-aminobenzoic acid, EDDnp is *N*-(2,4-dinitrophenyl)ethylenediamine], which were based on the human kininogen sequence at the C-terminal region of bradykinin. At the X position were inserted non-natural basic amino acids aiming at mapping the specificity of the S1 subsite of this enzyme. The longer peptides were more susceptible to KLK1, while in the shorter series the compound Abz-F-Ama-S-R-Q-EDDnp (**9**) (Figure 3), where Ama is 4-(aminomethyl)-cyclohexylalanine, inhibited KLK1 with a K_i of 50 nM with high specificity compared to human plasma kallikrein, thrombin, plasmin, and trypsin.[38]

The following series was designed based on phenylacetyl-Phe-Ser-Arg-EDDnp (structure not disclosed), a quite specific inhibitor for KLK1 that showed significant analgesic and anti-inflammatory effects. Phe was substituted by amino acids containing larger aliphatic or aromatic side chains as well as by unnatural basic amino acids, combining a large hydrophobic and/or aromatic group with a positively charged group at their side chains. The most effective competitive inhibitor for KLK1 was phenylacetyl-Aca-Ser-Arg-NH_2 (**10**), where Aca is L-*cis*/*trans*-4-(aminocyclohexyl)alanine, with a K_i of 0.11 μM.[120] Finally, the S'_1 and S'_2 subsite specificity of KLK1 was examined using the peptide Abz-Ala-Ile-Lys-Phe-Phe-Ser-Arg-Gln-EDDnp (structure not disclosed), corresponding to the reactive-center loop sequence of kallistatin, a serpin that displays high specificity for KLKs, being a strong inhibitor of KLK1 and KLK7, while KLK14 is slowly inhibited.[121] The information recovered with several derivatives of the reported sequence led to the synthesis of six shorter peptides that inhibited KLK1 in the range 20−30 nM. Among them acetyl-D-Lys-Phe-Phe-Pro-Leu-Glu-NH_2 (**11**) showed a surprising inhibitory potency, K_i of 8 nM, toward human plasma kallikrein, known to be a restricted arginyl hydrolase.[45] By screening of a phage display library, expressing 10 or 11 amino acids long linear peptides, six KLK2 binding peptides were identified. Among them, three compounds (corresponding to the sequences SRFKVWWAAG, AARRPF-PAPS, and PARRPFPVTA; structures not disclosed) were shown to be specific and efficient inhibitors of the enzymatic activity of KLK2. Alanine scanning of SRFKVWWAAG led to ARFKVWWAAG (structure not disclosed), which showed the strongest inhibition with a K_i of 1.41 μM.[122] On the basis of these results and to obtain more stable products for in vivo use, cyclic analogues were prepared and their biological activity and

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structural stability were compared.[123] Inhibition of KLK3/PSA is one of the major targets among the KLKs for medical and pharmaceutical research. Identification of highly selective peptide inhibitors, in fact, could be helpful for development both of novel pharmacological treatment and of targeted imaging agents for prostate cancer. Structure-guided approach has been employed for designing several peptides whose binding to PSA has been assessed by surface plasmon resonance (SPR) that showed strong binding affinity of PSA toward peptides with hydrophobic and basic residues.[124] Starting from the previously known PSA peptide substrate Ser-Ser-Lys-Leu-Gln (SSKLQ, structure not disclosed), a rational and iterative approach has been used to develop peptidyl boronic acid based PSA inhibitors. From these studies a peptidyl boronic acid with the sequence carbobenzyloxy-Ser-Ser-Lys-Leu-(boro)Leu (**12**) (Figure 4) has been identified as a potent and specific PSA inhibitor with a K_i of 65 nM.[125]

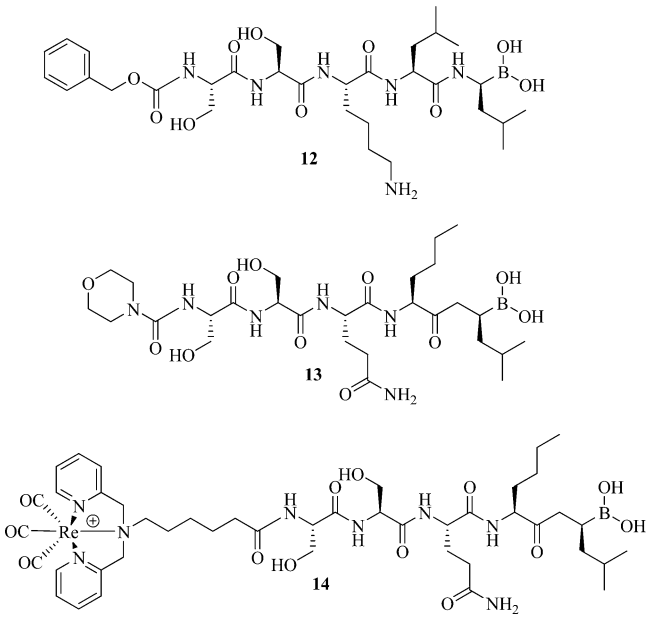

Figure 4. Peptide and peptide-mimetic KLK3/PSA inhibitors obtained by drug design approach.

A subsequent optimization led to the identification of the compound morpholinocarbonyl-Ser-Ser-Gln-Nle-(boro)-Leu (**13**) with a K_i of 25 nM and with the desirable property to be linked to a single amino acid chelate (SAAC) group. Labeling with Re afforded the inhibitor **14** functioning well as an inhibitor of PSA with a K_i of 28 nM.[126] A combination of molecular modeling and virtual positional scanning studies has been employed in order to define the binding mode of peptide substrates into the catalytic pocket of KLK3. The utility of the obtained molecular insights was demonstrated by synthesizing several peptide sequences containing aldehyde functionality at the C-terminal.[127] Finally, Juliano et al. have reported a detailed study on the effect of sodium citrate and glycosaminoglycans on the proteolytic activity of KLK3. These two substances have been chosen, since they are very concentrated in prostate and seminal fluid. The obtained results demonstrated that sodium citrate can modulate KLK3 activity, enhancing the hydrolytic efficiency at Arg, Gln, and particularly Pro residues, thus suggesting that promising inhibitors can be designed based on the structure of *o*-phenanthroline and from compounds derived of proline.[32] KLK4 represents another interesting target related to prostate cancer development and progression. A sparse matrix peptide library of 125 individual tetrapeptide-*p*-nitroanilides (pNAs) has been designed. The found optimal cleavage sequence has been substituted into the naturally occurring sunflower trypsin inhibitor (SFTI) scaffold, affording an inhibitor with a nanomolar K_i that is able to block stimulation of PAR activity.[128] Another natural occurring inhibitor used as template for drug design is LEKTI consisting of 15 inhibitory domains (D1−D15) separated by 14 spacing segments. All of them, except for D1, showed specific and differential inhibition of KLK5, KLK7, and KLK14 with the inhibitory domains D8−D11 showing the strongest inhibition toward KLK5.[129] Recently, the 68-residue LEKTI domain 6 has been produced by the fragment condensation approach and site-directed cystine bridge formation, inhibiting KLK5 equipotently to the recombinantly produced peptide.[130]

Small Molecule KLK Inhibitors. Most of the synthesized small molecules designed as serine protease inhibitors are characterized by the presence of peptidomimetic moieties that are able to bind some key groups in the specificity pockets of the enzyme associated with an altered "scissile" bond which usually may react with the catalytic serine. Several patents have been recently published related to aminothiazoles,[131] azaindoles,[132] and [6-aminopyridin-3-yl)methyl]amide derivatives as KLK1 inhibitors.[133] Heterocyclic compounds, inhibiting KLK3/PSA at submicromolar concentrations, were identified by a high-throughput screening approach. Benzoxazinone derivative **15** (Figure 5), with a K_i of 300 nM, and triazole derivative **16**, with a

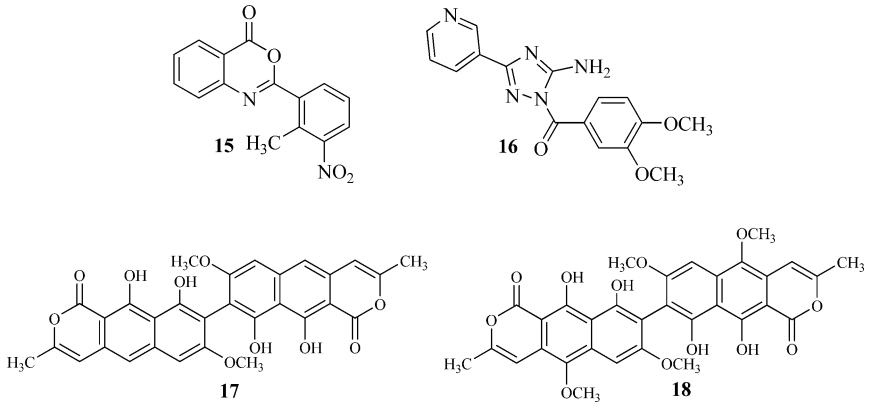

Figure 5. Small molecule KLK inhibitors.

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K_i of 500 nM, were used as lead compounds to perform a structure−activity relationship study.[134]

Some natural isocoumarins showed a promising inhibitory activity toward KLK5 and KLK7. In particular, vioxanthin (**17**) remains the most potent inhibitor of KLK5 (K_i = 22.9 μM), while 8,8′-paepalantine (**18**) showed a K_i of 12.2 μM toward KLK7.[135]

PAR AGONISTS AND ANTAGONISTS

Design and preparation of peptide-mimetics or small organic molecules acting on PARs have been very challenging, since the tethered ligand binding mechanism is energetically preferred. It is very difficult to compete against this intramolecular binding with a small-molecule ligand. Very few details about the three-dimensional structure of the receptors are available. This means that a highly empirical approach has been used so far. Several detailed overviews on PAR ligands have been published focusing on pathological conditions in which PARs are involved, such as cardiovascular diseases, inflammation, and tumor progression. In this section of the review we will analyze the most recent developments in the preparation and biological evaluation of PAR ligands. The classification of the molecules is based first on the specific target and second on the chemical structure. In particular we analyze (i) peptide/peptidomimetic PAR1 antagonists, (ii) small molecules as PAR1 antagonists, (iii) peptide-mimetic PAR2 agonists, (iv) small molecules as PAR2 agonists and antagonists, (v) peptide-mimetic PAR4 agonists and antagonists, (vi) small molecules as PAR4 antagonists, and (vii) pepducins as PAR4 antagonists.

Peptide/Peptidomimetic PAR1 Antagonists. Extensive structure−activity relationships studies, started using as model the sequence of the small peptides that selectively activates the thrombin receptor (SFLLRN or SFLLR), led to the identification of a "three-point model", constituted by the ammonium group, the center of the benzene ring (Phe residue), and the central carbon of the guanidine group (Arg residue). These features in conjunction with different molecular templates such as benzene, naphthalene, benzimidazole, indole, and indazole allowed the discovery of several potent peptide-mimetic PAR1 antagonists embodying 6-aminoindole (**19** and **21**) (Figure 6) and 6-aminoindazole (**20**) nuclei that met the spatial requirements for displaying the three key substituents in the correct three-dimensional configuration.[136−138]

Peptide-mimetic antagonists containing novel heterocyclic scaffolds, such as 2-methylindole and 1,4-benzodiazepine, were described. The pharmacological activity was assessed using human platelet aggregation induced by PAR1-AP in order to test the antagonist potency. Compound **22** (Figure 6) differs from **21** only because of the presence of a methyl group in position 2 of the 6-aminoindole moiety. This small structural modification shifted the biological activity toward a more potent derivative (IC_{75} analysis gave 0.1 μM for the reference compound **21** and 0.02 μM for **22**).[139] A different approach to receptor inhibition involved the use of N-palmitoylated peptides, termed pepducins, that, targeting the intracellular loops, can cause activation and/or inhibition of G protein signaling only in the presence of the parent GPCR. Attachment of a palmitate lipid to peptides based on the N-terminal or the C-terminal portion of the i3 loop of PAR1 yielded the full antagonist P1pal-12 (pal-RCLSSSA-VANRS-NH_2, structure not disclosed) or P1pal-7 (pal-KKSRALF-NH_2, structure not disclosed), respectively.[140,141] The latter, tested in xenograft models of advanced peritoneal ovarian cancer, reduced production of ascites and angiogenesis

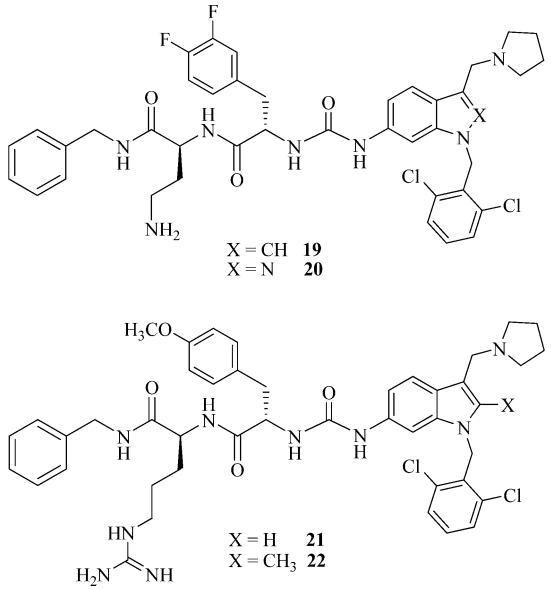

Figure 6. Peptide/peptidomimetic PAR1 antagonists.

and, when administered in combination with docetaxel, inhibited metastatic progression of peritoneal ovarian cancer.[89]

Small Molecules as PAR1 Antagonists. Several authors have described low molecular weight thrombin receptor antagonists resulting from the examination of structure−activity relationships performed on peptide-mimetic antagonists or from high-throughput screening. Among the non-peptide thrombin receptor antagonists the pyrroloquinazoline-based derivative **23** (SCH-79797) (Figure 7), first reported by Ahn et al. in 1999,[142] has shown an interesting cardioprotective effect related to its ability in inhibiting PAR1 attenuating myocardial injury and dysfunction related to myocardial I/R injury when given before or during ischemia.[143]

Testing in an in vivo angiogenesis model provided direct evidence that PAR1 is involved in the initiation of the angiogenic cascade.[144] **23** has shown a remarkable antiproliferative effect in several cell lines, even though the observation that it was able to slow the proliferation rate of mouse PAR1 null cells as well suggested that this pharmacological effect was likely not mediated by PAR1 inhibition.[145] PAR1 antagonist **24** (E-5555, named atopaxar), based on a bicyclic amidine motif, has been reported to inhibit platelet aggregation with no change in bleeding time in phase I studies, and it is currently under development as a potential treatment for critical care acute coronary syndrome. The safety and tolerability of **24** have been assessed in two parallel, international, phase II randomized, controlled studies: LANCELOT-CAD, conducted on 720 individuals with coronary artery disease (CAD), and LANCE-LOT-ACS, conducted on 603 patients who had experienced an acute coronary syndrome (ACS).[146,147] In the dose-ranging study of patients with CAD, treatment with **24** resulted in platelet aggregation inhibition, more minor bleeding, and numerically but not statistically fewer ischemic events. In patients after ACS, **24** significantly reduced early ischemia on Holter monitoring without a significant increase in major or minor bleeding. Larger-scale trials are needed to determine whether the results could be translated into clinically meaningful effects and to fully establish the efficacy and safety of **24**. In 2005, high affinity, orally active, low molecular weight non-peptide PAR1 antagonists, based on the natural product himbacine, were

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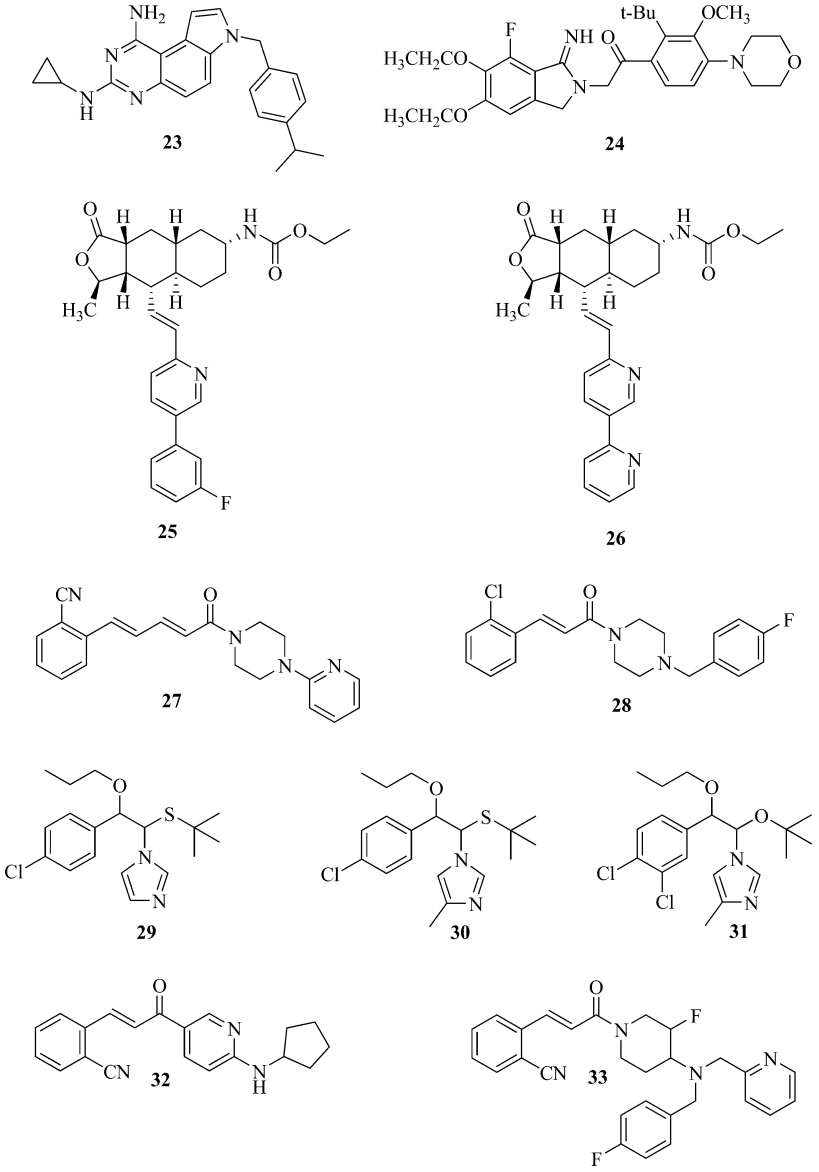

Figure 7. Small molecules as PAR1 antagonists.

reported.[148] Several structure−activity relationships studies were performed in order to optimize the substitution of important structural motifs for himbacine derivative PAR1 binding such as the lactone ring[149] and the pyridine ring.[150] Moreover, quinoline-based antagonists and other derivatives characterized by different tricyclic or heterotricyclic core were described.[151−153] Exploration of the C-7 region of the tricyclic motif led to a further optimization of himbacine-derived PAR1 antagonists, allowing the discovery of **25** (SCH-530348, named vorapaxar) (Figure 7), characterized by high potency in several in vitro functional assays and excellent oral bioavailability in multiple species.[154] Compound **25** has been evaluated in patients undergoing nonurgent percutaneous coronary intervention and was generally well tolerated. It did not cause increased thrombolysis in myocardial infarction (TIMI) bleeding, even when administered in association with aspirin and clopidogrel.[155] These results have allowed the design of a large phase III program, TRA·CER, a multicenter, randomized, double-blind, placebo-controlled study.[156] A part of this study, named TRA2°P-TIMI 50 trial, has been designed in order to evaluate the efficacy and safety of **25** during long-term treatment of patients with established atherosclerotic disease receiving standard therapy (up to 27 000).[157] At the beginning of 2011, Merck announced that **25** would be discontinued in patients who experienced a stroke prior to entry or during the trial because of an increase in intracranial hemorrhage in these patients. A more water-soluble analogue of **25** has been obtained through the incorporation of polar substituent on the pyridine ring. Compound **26** showed PAR1 affinity and antiplatelet effect in vivo in a cynomolgus monkey model comparable to those of vorapaxar with 20-fold increased aqueous solubility (K_i = 8 nM for **25** and K_i = 19 nM for compound **26**; kinetic solubility of <5 μM for **25** and 75 μM for compound **26**). In a Folts model of thrombosis, **26** showed dose-dependent antithrombotic efficacy that was additive when coadministered with the ADP antagonist cangrelor.[158] Perez et al. have reported phenylpentadienoylpiperazines and cinnamoylpiperazines able to inhibit SFLLR-induced human platelet aggregation and active by both iv and oral administration routes in a rat thrombosis model without any significant impact on bleeding time. Two of the described compounds, **27** (F16618)

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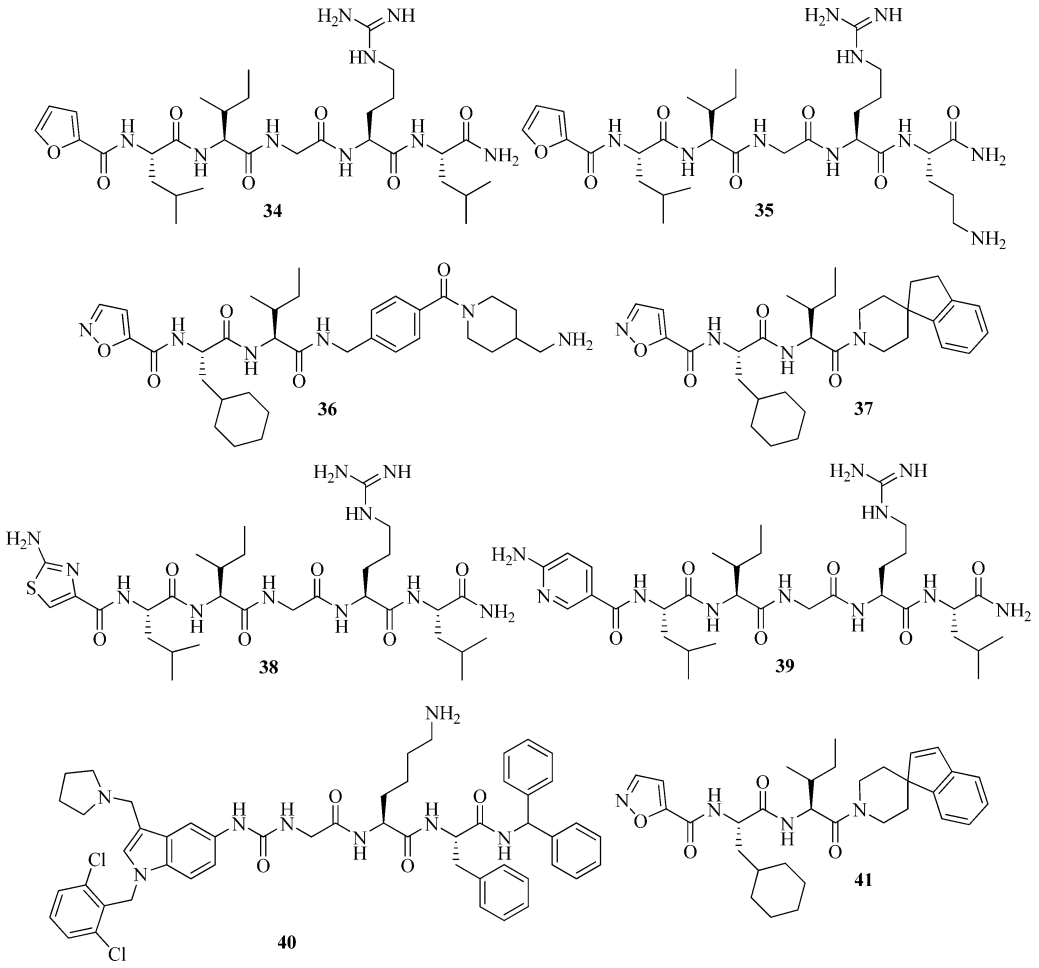

Figure 8. Peptide-mimetic derivatives as PAR2 agonists and antagonists.

and **28** (F16357) (Figure 7), are the most promising in terms of antithrombotic activity and ADME profile.[159] These compounds displayed antithrombotic activity in an arteriovenous shunt model in the rat. In particular, **27** exerted a potent antithrombotic activity by intravenous and oral routes, without affecting bleeding time. Furthermore, the antithrombotic activity was potentiated when combined with aspirin or clopidogrel.[160] Further pharmacological characterization has demonstrated that inhibition of PAR1, mediated by antagonists such as compound **27**, represents a highly effective treatment of restenosis after vascular injury, by inhibition of TNFα, MMP7, and smooth muscle cell migration and proliferation in addition to an antithrombotic effect.[161] As a consequence of an in-house high-throughput screening program, the same research group has identified a novel inhibitor hit, **29** (Figure 7), that was synthesized as a mixture of enantiomers. Compound **29** has been used as starting point for a SAR study. After modifications of the ether substituent, of the aryl group, of the imidazole ring, and of the thioether functionality, compounds **30** and **31**, tested as mixtures of enantiomers, were identified displaying an increase in potency with respect to **29** and strong antithrombotic activity in vivo associated with a sufficient stability against human liver microsomes.[162] Two series of cinnamoylpiperidine and cinnamoylpyridine derivatives were described as a result of profound structural modifications of the lead **28**. Several substitutions have been performed, such as incorporation of a fused phenyl−alkene system, replacement of the central carbonyl moiety by the known cyclobut-3-ene-1,2-dione bioisostere, and exploration of the piperazine portion that led to the most active compounds such as the pyridine analogue **32**, which revealed a very comparable pharmacological profile with respect to the reference compound **28**, and piperidine analogue **33**, displaying a significantly improved activity in the rat antithrombotic model.[163]

Peptide-Mimetic PAR2 Agonists and Antagonists. Research of PAR2 receptor ligands has been directed toward identification of agonists and antagonists, since both of them may have therapeutic value. PAR2 antagonists are of particular interest in order to relieve, for example, inflammatory symptoms in rheumatoid arthritis.[88] On the other hand, activation of PAR2 could play a relevant role as gastric cytoprotective and/or airway smooth muscle relaxation mediator.[164] Research for PAR2 ligands, based on chemical modifications of short peptides corresponding to the newly exposed N-terminus of the receptor, such as PAR2-AP, led to more potent agonists, namely, 2-furoyl-LIGRL-NH_2 (**34**) and 2-furoyl-LIGRO-NH_2 (**35**) (Figure 8).[165,166]

Starting from these compounds, Barry et al. has reported a small compound library based on a XLIGRLI-NH_2 hexapeptide motif. Some chemical modifications have been performed such as heterocyclic replacement for the N-terminal serine, truncation and optimization of very short peptides, and then substitution of the C-terminus with nonpeptidic fragments. Among the reported derivatives, agonist **36** (GB110), showing an EC_{50} of 0.28 μM, selectively induced PAR2-mediated, but not PAR1-mediated, intracellular Ca^{2+} release in HT29 human colorectal carcinoma

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cells, while antagonist **37** (GB83), with an IC_{50} of 2 μM, is the first compound at micromolar concentrations to reversibly inhibit PAR2 activation by both proteases and other PAR2 agonists.[167] Despite the interesting activity of these derivatives in many PAR2 cellular and molecular studies, the furane ring cannot be considered as a safe structural element for drug design, since it can be metabolically activated by cytochrome P450 to form electrophilic intermediates that are able to bind covalently to liver proteins and cause hepatotoxicity in vivo. For these reasons Boitano et al. have designed and synthesized a set of 15 analogues to investigate the effects of N-terminal modifications on the ability to activate PAR2. They found that the most potent agonists share a common structural feature, nitrogen based heterocycle (thiazole or pyridine) with an amino group orientated in the position ortho to the nitrogen. In particular 2-aminothiazol-4-yl and 6-aminonicotinyl derivatives (**38** and **39**) were discovered as selective PAR2 agonists.[168] A further pharmacological characterization of compounds **35**, **38**, and **39** have been reported showing that all of them are able to stimulate PAR2-dependent in vitro physiological responses, MAPK signaling, and Ca^{2+} signaling with an overall rank order of potency of **35** ~ **38** > **39** ≫ SLIGRL-NH_2. Moreover, their evaluation in models relevant to nociception showed that all three agonists activated Ca^{2+} signaling in nociceptors in vitro, and both **35** and **38** stimulated PAR2-dependent thermal hyperalgesia in vivo.[169] From the screening of a peptide-mimetic compound library based on the structures of PAR2 agonist peptides, compound **40** (K-14585) was identified as able to inhibit PAR2-dependent calcium and proinflammatory signaling but not attenuating MAPK signaling acting as a partial PAR2 agonist.[170,171] A recent attempt to optimize the pharmacological profile of compound **37** has led to the identification of a more potent small molecule peptide-mimetic antagonist, **41** (GB88, Figure 8).[172] This compound, evaluated for blockade of intracellular calcium release in colonocytes and for anti-inflammatory activity in acute (PAR2 agonist-induced) versus chronic (TNBS-induced) models of colitis in Wistar rats, provided the first evidence that in vivo pharmacological targeting of PAR2 with an antagonist attenuates experimental colitis.[173]

Small-Molecule Compounds as PAR2 Agonists and Antagonists. The development of small-molecule non-peptide PAR2 agonists and antagonists has proved to be challenging. From the screening of a chemical library containing more than 250 000 small-molecule druglike compounds a small number of active structures were identified. Agonist activity at the human PAR2 was evaluated utilizing a high-throughput functional screening, such as R-SAT. A focused library around **42** (AC-55541, Figure 9), made by reacting a number of aromatic aldehydes or ketones with the parent hydrazide, led to the preparation of the more active derivative **43** (AC-264613).[174]

Compounds **42** and **43** have been characterized in vitro and in vivo, displaying high potency and efficacy for PAR2 receptors in a variety of functional assays including cellular proliferation, calcium mobilization, and PI hydrolysis assays, and both compounds stimulated internalization of PAR2 receptors.[175] Very few small molecule PAR2 antagonists have been described to date. The piperazine derivative **44** (ENMD-1068, Figure 9), based on the sequence LIGK-NH_2, has shown the ability to block trypsin and PAR2-AP activation of the receptor and, despite its very low potency, has proved of use in in vivo studies.[88] The obtained results allowed PAR2 antagonism to be identified as a potentially powerful strategy for treatment of arthritis.

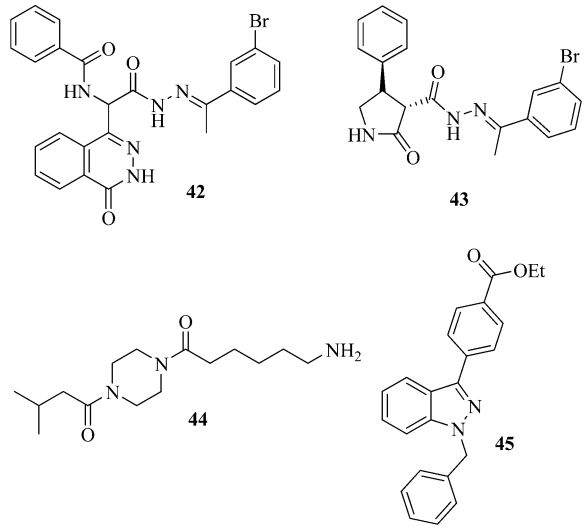

Figure 9. Small-molecule compounds as PAR2/PAR4 agonists and antagonists.

Small-Molecule Compounds and Pepducin-Based PAR4 Antagonists. PAR4 antagonism can be considered an interesting strategy in order to get antithrombotic agents, since PAR4 receptors are expressed along with PAR1 on human platelets. However, only few molecules have been reported acting on this receptor subtype. Among them, the indazole derivative **45** (YD-3, Figure 9) inhibited thrombin-stimulated murine platelet aggregation.[176] However, in human platelets, which also express PAR1, this compound blocks platelet aggregation induced by the PAR4 peptide agonist GYPGKF-NH_2 but not by thrombin. The application of the pepducin approach to the search of PAR4 antagonists has led to the identification of a selective PAR4 antagonist **46** (structure not disclosed), named P4pal10, whose sequence is pal-SGRRYGHALR-NH_2.[140,141] This compound completely inhibits platelet aggregation, prolongs bleeding time, and prevents systemic platelet activation in mice.[177] **46** and a structurally unrelated PAR4 antagonist, *trans*-cinnamoyl-YPGKF-NH_2 (structure not disclosed), showed a cardioprotective effect due to the unmasking of adenosine receptor signaling, supporting the hypothesis of a coupling between thrombin and adenosine receptors.[178] The effects of PAR4 inhibition have been tested in a model of systemic inflammation and disseminated intravascular coagulation (Shwartzman reaction), furnishing circumstantial evidence that the primary cellular target of **46** may be neutrophils rather than platelets or endothelial cells. This finding suggests a possible development for PAR4 antagonists in the treatment of systemic inflammation.[179]

■ CONCLUSIONS

Kallikrein-related peptidases (KLKs) are a family of serine proteases that are involved in many biological functions. KLK activity needs to be tightly regulated by means of several modulators such as ions and reversible/irreversible protein inhibitors. Many pathological conditions in humans arise from a failure in controlling KLKs activity. Several members of the family have been found to be involved in pathological conditions such as cancer and inflammation affecting different tissues. Protease activated receptors (PARs) have been recently identified as KLK substrates in many different physiological and pathological settings. Several pharmacological studies have

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then proposed the PAR−KLK axis as a useful pharmacological target for drug research. Interesting results have been obtained in the field of PAR antagonists, in particular with the molecules that are able to block PAR1 receptor. Most of the pharmacological studies involve their employment in cardiovascular diseases. In the future, attention should be also addressed to the evaluation of PARs antagonists in disease models where KLKs play a pivotal role exerting their actions through PAR activation.

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Notes

The authors declare no competing financial interest.

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Giuseppe Caliendo received his B.Sc. in Pharmacy from University of Naples Federico II, Italy, in 1981. Currently, he is Full Professor of Medicinal Chemistry at the Faculty of Pharmacy of Naples. He has published over 120 peer-reviewed papers and some scientific books in the field of peptidomimetics and synthesis by microwave of biologically active compounds. His research is focused on identification and characterization of heterocyclic derivatives acting on some of the most attractive targets for medicinal chemists such as serotonin receptors, protease activated receptors, and kallikreins. In recent years, he has been interested in the development of hybrid structures containing H_2S-releasing moiety in order to produce novel pharmacological tools able to highlight the therapeutic potential of modulation of the H_2S pathway.

Vincenzo Santagada received his B.Sc. in Pharmacy from University of Naples Federico II, Italy, in 1982 and his Ph.D. in Medicinal Chemistry in 1987. Currently, he is Full Professor of Medicinal Chemistry at the Faculty of Pharmacy of Naples. He has published over 120 peer-reviewed papers and some scientific books in the field of peptidomimetics and synthesis by microwave. His scientific interests cover design and synthesis of pharmacologically active peptides and peptidomimetics that have been evaluated for their antitumoral, anti-inflammatory, antiviral, or analgesic properties. Recent years have seen a deep interest in the application of microwave technology to medicinal chemistry and in the development of H_2S-releasing molecules.

Elisa Perissutti obtained her B.Sc. in Pharmacy from University of Naples Federico II in 1984 followed by a Ph.D. in Pharmaceutical Sciences in 1989. Currently she is a member of the Department of Pharmaceutical and Toxicological Chemistry and is Associate Professor of Medicinal Chemistry at the Faculty of Pharmacy. She has published over 70 full articles in international journals. Her main scientific interests include structure−activity relationship studies on heterocyclic derivatives acting as serotoninergic ligands, identification and characterization of PAR ligands, and application of novel technological tools to drug discovery. She is also coauthor of a book on the applications of microwave in peptide and organic synthesis.

Beatrice Severino obtained her Bachelor's degree in Medicinal Chemistry in 1997 from University of Naples Federico II, Italy. In 2003 she earned a Ph.D. in Pharmaceutical Science, working on a project focused on the design and synthesis of peptidomimetic derivatives acting on protease-activated receptors. In 2006 she held the position of Assistant Professor in Medicinal Chemistry at Faculty of Pharmacy, where she serves as a full-time researcher. Her scientific interest is mainly focused on development of peptidomimetic derivatives acting on several pharmacological targets such as PARs, kallikreins, and more recently sphingosine kinases. Her results are documented in 49 publications in international journals.

Ferdinando Fiorino graduated from University of Naples Federico II, Italy, with a degree in Pharmacy in 1995. His interest in medicinal chemistry started at the Department of Pharmaceutical and Toxycological Chemistry, and he received his Ph.D. in Pharmaceutical Science in 1999 from University of Naples Federico II. In 2005 he held the position of Assistant Professor in Medicinal Chemistry at Faculty of Pharmacy, where he serves as a full-time researcher. His research has focused on the synthesis and structure−activity relationships of heterocyclic compounds with serotonergic activity in order to obtain ligands with greater potency and selectivity on 5-HT1A receptors. More recently he has devoted his attention to the synthesis and structural characterization of peptide fragments of prion protein in order to clarify the mechanism by which the physiological form is converted to the pathological form. He is author of 50 publications in international journals.

Francesco Frecentese graduated from University of Naples Federico II, Italy, with a degree in Pharmacy in 2003. Then he received his Ph.D. in Pharmaceutical Science in 2006 from University of Naples Federico II, working on a project concerning the application of microwaves in the synthesis of pharmacologically active heterocyclic, peptidic, and peptidomimetic molecules. He is currently a full-time researcher at Faculty of Biotechnology, and his interest is focused on development of PAR ligands and agents acting as H_2S releasing molecules and/or inhibitors of enzymes involved in H_2S production pathway. His scientific results are reported in 21 articles in international journals.

Luiz Juliano received his M.D. degree in Medicine in 1968 and his Ph.D. in Biological Science−Molecular Biology in 1974 from the Federal University of São Paulo, Brazil. Currently is a Full Professor at the same university. Luiz Juliano has experience in the area of biochemistry, with emphasis on proteins, working mainly on themes related to proteases and biological active peptides.

ABBREVIATIONS USED

Abz, *o*-aminobenzoic acid; Aca, L-*cis*/*trans*-4-(aminocyclohexyl)-alanine; ACS, acute coronary syndrome; Ama, 4-(aminomethyl)-cyclohexylalanine; CAD, coronary artery disease; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; hK, human kallikrein; HMWK, high molecular mass kininogen; ICAM-1, intercellular adhesion molecule 1; KLK, kallikrein-related peptidase; KLKB1, plasma kallikrein; KOLF-PAR1, knockout mouse lung fibroblast cell line transfected with protease activated receptor 1; KOLF-PAR2, knockout mouse lung fibroblast cell line transfected with protease activated receptor 2; LEKTI, lymphoepithelial Kazal-type inhibitor; LMWK, low molecular mass kininogen; MBP, myelin basic protein; NS, Netherton syndrome; PAR, protease activated receptor; PAR-AP, protease activated receptor activating peptide; pNA, tetrapeptide-*p*-nitroanilide; pro-uPA, zymogen of urokinase plasminogen activator; PSA, prostate-specific antigen; R-SAT, receptor selection and amplification technology; SAAC, single amino acid chelate; SBTI, soybean trypsin inhibitor; SERPIN, serine proteinase inhibitor; SFTI, sunflower trypsin inhibitor; SPINK5, serine protease inhibitor Kazal-type 5; SPR, surface plasmon resonance; TGF-β, tissue growth factor type β; TIMI, thrombolysis in myocardial infarction; TNBS, trinitrobenzene sulfonic acid; TSLP, thymic stromal lymphopoietin; VIP, vasoactive intestinal peptide

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