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# Protease-activated receptor-2 (PAR-2): a potential new target in arthritis

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**Abstract**

Protease-activated receptors (PARs) are a novel family of seven-transmembrane G-protein-coupled receptors. The unique feature of this family is that activation is initiated by cleavage of the *N*-terminus by serine or other proteases, thereby unmasking a tethered ligand that then interacts with the receptor, leading to activation. PARs have been described in the context of inflammation, and recent evidence indicates a particular role for the second member of this family, PAR-2, in arthritis. Synovial expression of this receptor is greatly upregulated in murine models of arthritis, and both acute and chronic experimental monoarthritis are substantially attenuated in *Par2* knockout mice, suggesting a key role for PAR-2 in inflammatory joint disease. These findings translate to inflammatory disease in humans, since PAR-2 expression is upregulated in synovial tissues from patients with rheumatoid arthritis (RA), and appears to be an upstream regulator of proinflammatory cytokine generation, including tumor necrosis factor $\alpha$ (TNF-$\alpha$). These findings identify PAR-2 as a new therapeutic target in the management of RA, and the challenge is now to develop potent and selective agents to prevent activation of this receptor.

## Introduction

Protease-activated receptors (PARs) are a novel family of G-protein-coupled receptors originally cloned from murine and human sources (1-3), with a unique mode of activation involving a 'tethered' ligand (Fig. 1). Proteolytic cleavage of the *N*-terminus by specific serine proteases generates a new *N*-terminus. This forms a tethered ligand which activates the receptor, resulting in signaling and then internalization of the receptor complex, as previously reviewed (4-6). PAR-1 was the first of this receptor family to be discovered (7), and was originally called the thrombin receptor due to preferential activation by this enzyme. Since then, the family has expanded to include PAR-2, -3 and -4 (4-6). Similar to PAR-1, both PAR-3 and PAR-4 are preferentially activated by thrombin. In contrast, PAR-2 is relatively insensitive to thrombin and is preferentially activated by trypsin and related serine proteases, including mast cell tryptase (1, 8, 9), as well as other proteases such as neutrophil proteinase 3 (PR-3) or human leukocyte elastase (10). Interestingly, PAR-2 can also be activated by proteases originating from pathogenic organisms (11), suggesting that this receptor could play a role in innate immunity (12). There is also evidence to show that PAR-2 can be activated by some members of the tissue kallikrein family, with a potential role in cancer progression (13).

PARs can also be exogenously activated by peptide-derived ligands based on the amino acid structure of the tethered ligand, offering greater specificity than serine proteases, which potentially could affect non-PAR pathways. Modification of these peptide sequences has permitted the development of novel agonists with greater potencies. In the case of PAR-2, replacement of the serine on the amino terminal of the human tethered ligand sequence SLIGKV-OH with a furoyl group generated the more potent peptide 2f-SLIGKV-OH (14). The most potent PAR-2 agonist peptide developed to date (2f-LIGRL-$NH_2$) combines the furoyl group substitution within the amidated mouse tethered ligand sequence (15). In conjunction with inactive or scrambled peptide sequences, PAR-activating peptides can be used to selectively probe the function of these receptors.

Antagonists are also an important means of probing receptor function, but unfortunately for PAR-2, these have

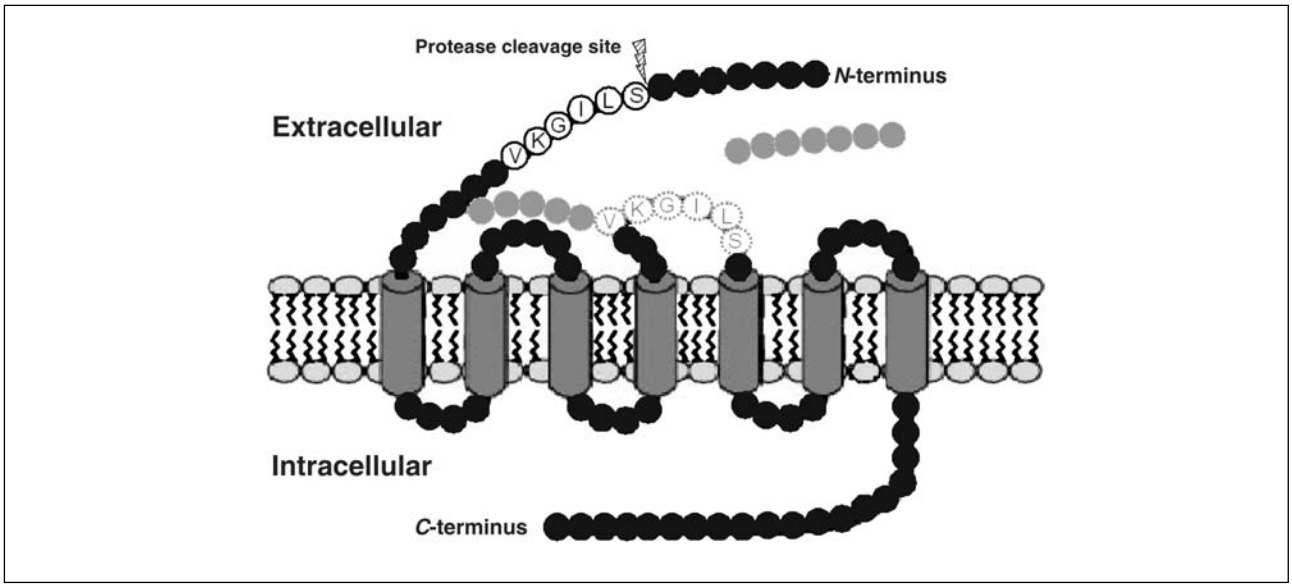


Fig. 1. Activation of human PAR-2. A serine protease, such as trypsin, cleaves the *N*-terminus, exposing the tethered ligand sequence (SLIGKV). The 'unmasked' sequence (gray) then interacts with extracellular loop 2, leading to signaling at the *C*-terminus via G-proteins. Administration of an exogenous agonist peptide that mimics the tethered ligand sequence can also lead to activation of the receptor (modified from Ref. 25 with permission).

proved difficult to generate. One recently developed, the small-molecule compound $N^1$-3-methylbutyryl-$N^4$-6-aminohexanoylpiperazine (ENMD-1068), is selective for PAR-2 but has low affinity (16). In addition, some peptide-derived compounds may not act as true receptor antagonists. Although trypsin-induced PAR-2 activation by an agent consisting of partial reverse PAR-1- and PAR-2-derived peptide sequences (FSLLRY-$NH_2$) was blocked (without affecting the proteolytic activity of trypsin), the vasodilating response to a PAR-2-activating peptide in the aortic ring preparation was unaffected (17). In the absence of potent and specific PAR antagonists, the use of *Par2* knockout mice has proved invaluable for establishing the specificity of action of PAR-2-activating peptides. Although not an exhaustive list, Table I provides selected examples of agents used to investigate PAR-2 functionality.

While PAR-2 is activated by some serine proteases, others can in fact 'de-activate' or disarm the signaling mechanism by cleavage and removal of the *N*-terminal sequence 'downstream' (*i.e.*, towards the *C*-terminus) of the tethered ligand, while the receptor remains functional, as signaling still occurs using the PAR-2-activating peptide SLIGKV-$NH_2$ (23, 24). This picture is further complicated by serine proteases such as human leukocyte elastase, which can both activate (10) and disarm (23) PAR-2. However, this could be related to differences in the cell types used, as PAR-2-expressing human gingival fibroblasts were activated (10) while KNRK/PAR-2, A549 and 16HBE cell lines were disarmed (23). Activation of PAR-2 by serine proteases can also be influenced by receptor glycosylation. Compton *et al.* (25) found that calcium signaling in PAR-2-expressing CHO cells in response to tryptase could be enhanced by deletion of the *N*-terminal glycosylation sequon on PAR-2, although the responses to trypsin and SLIGRL-$NH_2$ were unaffected.

*Table I: Examples of PAR-2 activators and inhibitors used in experimental investigations.*

| Activators | | Ref. |
|---|---|---|
| *Proteases* | Trypsin | 1 |
| | Tryptase | 7, 8, 18 |
| | Acrosin | 19 |
| | Factors VIIa and Xa | 20 |
| | Neutrophil proteinase 3 | 10 |
| | Kallikreins | 12 |
| *Agonists* | SLIGRL-$NH_2$ (mouse, rat) | 1 |
| | SLIGKV-$NH_2$ (human) | 3 |
| | 2-Furoyl-LIGKV-OH (ASKH95) | 14 |
| | 2-Furoyl-LIGRLO-NH2 (FLIGRL) | 21 |
| Inhibitors | | |
| | FSLLRY-$NH_2$ and LSIGRL-$NH_2$ | 17 |
| | *Solanum melongena* | 22 |
| | SAM11 | 16 |
| | B5 | 16 |
| | ENMD-1068 | 16 |

## PAR-2 as an activator of proinflammatory pathways

Evidence indicating that PAR-2 could mediate inflammatory responses arises from the observation that it is expressed in cells of the immune system, as well as 'interface' cells such as respiratory epithelium, enterocytes, keratinocytes and endothelial cells (6, 26-31). PAR-2 activation of such cells can evoke the synthesis of cytokines and prostaglandins, as well as the activation of the NF-κB and MAP (mitogen-activated protein) kinase

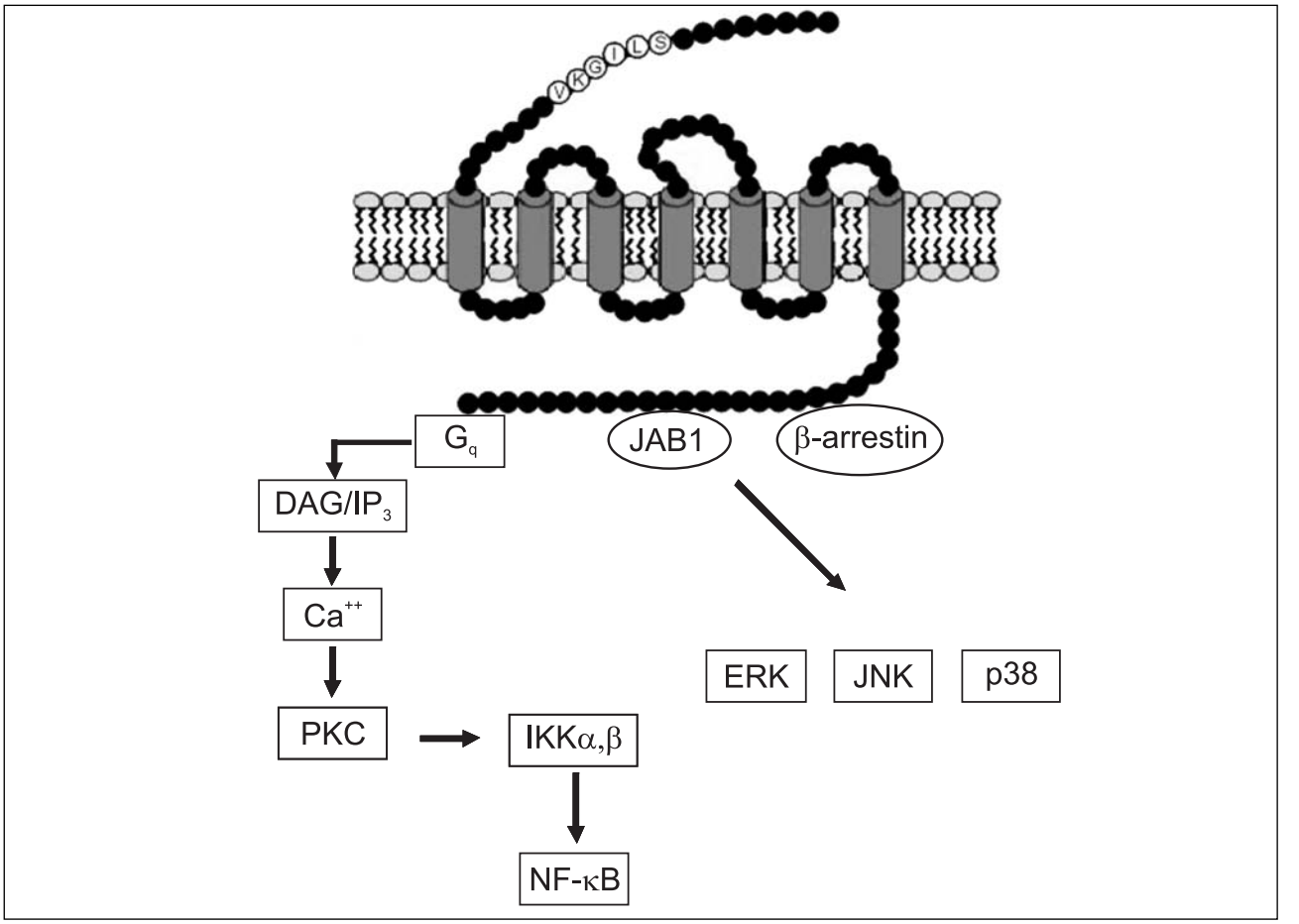


Fig. 2. PAR-2 signaling pathways. Activation of $G_q$ can activate the NF-κB pathway via the release of calcium from intracellular stores. In contrast, activation of MAP kinase is not calcium-dependent, but involves receptor interaction with specific proteins.

signaling pathways (27, 31-34). While PAR-2 activation of the NF-κB pathway is strongly dependent on $Ca^{2+}$-dependent protein kinase C (PKC) isoforms (34, 35), this is not the case for MAP kinases (36), coupling being more likely to occur via a direct interaction of the receptor with specific interacting proteins, such as JAB1 (37) and β-arrestins (38). Activation of specific signaling pathways is likely to be cell-dependent because, although the NF-κB signaling pathway is activated by PAR-2 agonists in human endothelial cells (31), in human chondrocytes SLIGKV-$NH_2$ activates the extracellular signal-regulated kinase ERK1/2 and p38 but not the Janus kinase (JNK) or NF-κB pathways (39). Nevertheless, irrespective of specific signaling mechanisms or cell types, these observations indicate that PAR-2 has the potential to mediate inflammatory responses and the cognate proinflammatory signaling pathways are indicated in Figure 2.

Experimental observations indicate that PAR-2 has proinflammatory actions in the joint, as intra-articular injection of SLIGRL-$NH_2$, a peptide-derived ligand based on the structure of the murine *N*-terminus that selectively activates PAR-2, results in knee joint swelling. This response was found to be enhanced when a modified PAR-2 agonist (2f-SLIGKV-OH) was used, but this effect was absent in *Par2* knockout mice. This provides evidence for the selectivity of such agents, further supported by the observation that administration of an inactive control peptide also failed to elicit joint swelling (14). Furthermore, topical application of this or other synthetic PAR-2 agonists to the murine synovium results in dose-dependent vasodilatation, and again this effect is absent in mice with a deleted *Par2* gene (14, unpublished observations). Interestingly, intra-articular injection of tryptase β also elicits dose-dependent joint swelling and synovial hyperemia in wild-type mice, but has no effect in *Par2* knockout mice (40), suggesting that perhaps some serine proteases act exclusively via this receptor in the joint.

The proinflammatory effects of PAR-2 are not confined to large joints, as intraplantar injection of a PAR-2 agonist leads to paw swelling and neutrophil infiltration in rodents (41, 42). These studies suggest that PAR-2 activation elicits cardinal signs of inflammation, making it a likely candidate pathway for mediating inflammatory responses in the joint, although there is evidence to suggest that in some tissues, such as lung (28), intestine (43) and gastric mucosa (44, 45), it may exert protective effects. However, even in these systems some experimental models of inflammation appear to be PAR-2-

dependent. Eosinophil infiltration and airways hyperreactivity due to ovalbumin-induced inflammation of the airways are substantially reduced in PAR-2-deficient animals but exacerbated in mice overexpressing PAR-2 (46). Similarly, indices of inflammation in infectious colitis are greatly reduced in PAR-2-deficient mice (47). Such differential effects may be an indication of the dual nature of PAR-2, with both physiological (antiinflammatory) and pathological (proinflammatory) roles. Indeed, this may be similar to other receptor systems which normally have a physiological, homeostatic function but can be subverted in diseases states.

## PAR-2 in the pathogenesis of experimental arthritis

Intra-articular injection of a carrageenan/kaolin mixture in mice results in an acute inflammatory response; compared to wild-type littermates, joint swelling in this model of acute arthritis was substantially reduced (> 80%) in PAR-2-deficient mice (16). This finding, together with the experiments described earlier where intra-articular injection of PAR-2-activating peptides led to joint swelling and perfusion, supports a proinflammatory role for this receptor in acute arthritis. More importantly, chronic monoarthritis using Freund's adjuvant was markedly reduced in PAR-2-deficient mice (14). Various indicators of the inflammatory process, such as joint swelling, pannus formation, cartilage degradation and bone invasion, were substantially attenuated in PAR-2-deficient mice. Interestingly, mice heterozygous for *Par2* showed an intermediate inflammatory phenotype. In the *Par2* knockout mice used in this study, the gene was disrupted by insertion of a *lacZ* reporter cassette, bringing expression of β-galactosidase under the control of PAR-2 transcriptional regulatory elements. Staining for β-galactosidase activity can thus be used to monitor expression of the targeted (disrupted) *Par2* allele. Using this approach, β-galactosidase (PAR-2) expression in uninflamed joints of PAR-2-deficient mice was found to be confined to small arterioles, whereas in chronically inflamed joints there was substantial β-galactosidase (PAR-2) upregulation, mostly occurring among various cell types in the extravascular compartment.

These observations clearly suggest that PAR-2 plays an important role in chronic arthritis and were recently confirmed in another study using methylated bovine serum albumin (mBSA) to elicit antigen-induced arthritis. In addition to a reduction in technetium uptake by the joint, histological scores of disease activity were also lower in PAR-2-deficient mice. In addition, these mice showed lower levels of anti-mBSA-specific immunoglobulin isotype $IgG_{2b}$, as well as reduced proliferation of lymphocytes from draining lymph nodes stimulated *in vitro* with mBSA (48). These immunological data are interesting, as there is evidence that the maturation of murine dendritic cells (DCs) is PAR-2-dependent (49). DC maturation is a critical step in the initiation of an immune response, suggesting a role for PAR-2 in the immune response during arthritis.

Although the studies described above indicate PAR-2 involvement in arthritis, this has not been the case for all models of experimental arthritis. K/BxN serum-induced arthritis and zymosan-induced arthritis were unaffected in PAR-2-deficient mice (48). In this context, it is important to establish the role of PAR-2 in the collagen-induced arthritis (CIA) model, generally regarded as the experimental rodent model best mimicking rheumatoid arthritis (RA). A difficulty in testing this model arises, as the *Par2* knockout mouse is based on the C57Bl/6 background which is resistant to commonly used protocols for developing CIA, whereas DBA1 mice are the most susceptible strain. However, using the PAR-2 antagonist ENMD-1068 we have recently been able to demonstrate a reduction in the arthritis index using the CIA model in the DBA1 strain (50).

Data concerning the role of other PARs in arthritis are more limited. CIA in mice is reduced when the preferential PAR-1 activator thrombin is inhibited (51), while murine adjuvant-induced arthritis shows significantly reduced severity in PAR-1-deficient mice, associated with a decrease in synovial inflammatory cytokine levels (52). However, these effects are relatively small, and to date, no studies have examined the potential role of either PAR-3 or PAR-4 in chronic models of arthritis, nor the interaction among different PARs.

## PAR-2 in the pathogenesis of rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease of unknown etiology that affects approximately 1% of the population (53). Multiple cell types are involved, including lymphocytes, granulocytes, monocytes and mesenchymal cells, particularly fibroblast-like synoviocytes (54, 55). The cardinal feature of the disease is proliferative inflammation of the synovial membrane affecting multiple joints, resulting in destruction of the adjacent cartilage and bone. Although the etiology and pathogenesis of RA remain obscure, there is evidence indicating a potential role for PAR-2 in its pathogenesis.

PAR-2 is expressed in the RA synovium, most clearly observed in the lining and sublining areas, as well as in blood vessels (48, 56). The lack of availability of uninflamed synovium from healthy subjects as a control makes evaluation of the significance of PAR-2 expression in RA difficult, and therefore synovium from patients with osteoarthritis (OA) is commonly used as a comparator control. This is based on the long-standing view that OA is a degenerative disease without an inflammatory component; however, this may be a misperception, as there is now emerging evidence to suggest that synovitis may be present in OA (57). Nevertheless, judicious selection of OA samples not demonstrating inflammatory features can be used for control purposes. PAR-2 expression in such control samples is substantially less than in RA samples (48, 56). Interestingly, despite marked synovitis, there is almost a complete absence of PAR-2 expression in synovial tissue samples obtained from patients with psoriatic arthritis (56), indicating that inflammation *per se* may not drive PAR-2 expression, and pointing to different inflam-

matory pathways being involved in psoriatic arthritis compared to RA.

While this review is focused on RA, it is interesting to note that it has recently been discovered that PAR-2 may play a role in the pathogenesis of OA. This receptor is upregulated in OA chondrocytes and a selective PAR-2-activating peptide (SLIGKV-$NH_2$) resulted in generation of matrix metalloproteinases 1 (MMP-1) and 13 (MMP-13) and cyclooxygenase type 2 (COX-2) from cartilage explants (39).

It has been possible using specific cell markers in RA synovial samples to demonstrate PAR-2 expression in fibroblasts, macrophages, mast cells, as well as B- and T-cells, cell types known to contribute to the pathogenesis of RA. Fibroblast-like synoviocytes have also been examined for PAR-2 expression, and again there is a clear difference between RA and OA, the former showing much greater expression, both immunohistochemically (48, 56) and by Western blotting (56). However, analysis using RT-PCR has tended to yield more variable results among different research groups. While Busso *et al.* (48) found clear evidence of PAR-2 mRNA in RA but not OA fibroblast-like synoviocytes, Abe *et al.* (58) found almost a complete absence of PAR-2 mRNA in RA (PAR-1 and -3 being much greater), although this was upregulated by basic fibroblast growth factor (bFGF). Although these data show an association between PAR-2 expression and RA, they do not prove causality. However, functional data recently obtained provide strong support for this relationship, as generation of tumor necrosis factor $\alpha$ (TNF-$\alpha$) by RA synovial membrane was substantially blunted by the PAR-2 antagonist ENMD-1068 (56). A significant reduction in the generation of other proinflammatory cytokines such as interleukin-1$\beta$ (IL-1$\beta$) was also observed.

### Therapeutic potential of inhibiting PAR-2 activation

There are a number of potential approaches to prevent PAR-2 activation, ranging from genomic intervention through direct receptor antagonism to intervention in intracellular signaling pathways (Fig. 3). We previously demonstrated that gene silencing using PAR-2 small interfering RNA (siRNA) was effective at reducing acute joint inflammation induced by intra-articular injection of a carrageenan/kaolin mixture. Similarly, serine protease inhibitors were effective antiinflammatory agents in this model. Prevention of proteolytic activation of PAR-2 using antibodies targeting the serine protease cleavage site also inhibited joint swelling. Finally, direct receptor antagonism using the PAR-2 antagonist ENMD-1068 dose-dependently inhibited joint swelling (16). These findings provide proof of concept that inhibiting PAR-2 is an effective antiinflammatory strategy, and there are a number of possible ways to achieve this. Although these studies were performed in a murine model of acute arthritis, they translate to RA, as TNF-$\alpha$ generation from RA synovial membrane was potently inhibited by ENMD-1068 (56). Interventions involving interruption of signaling pathways in the inflammatory cascade are also possible, although this requires agents able to penetrate the cell membrane.

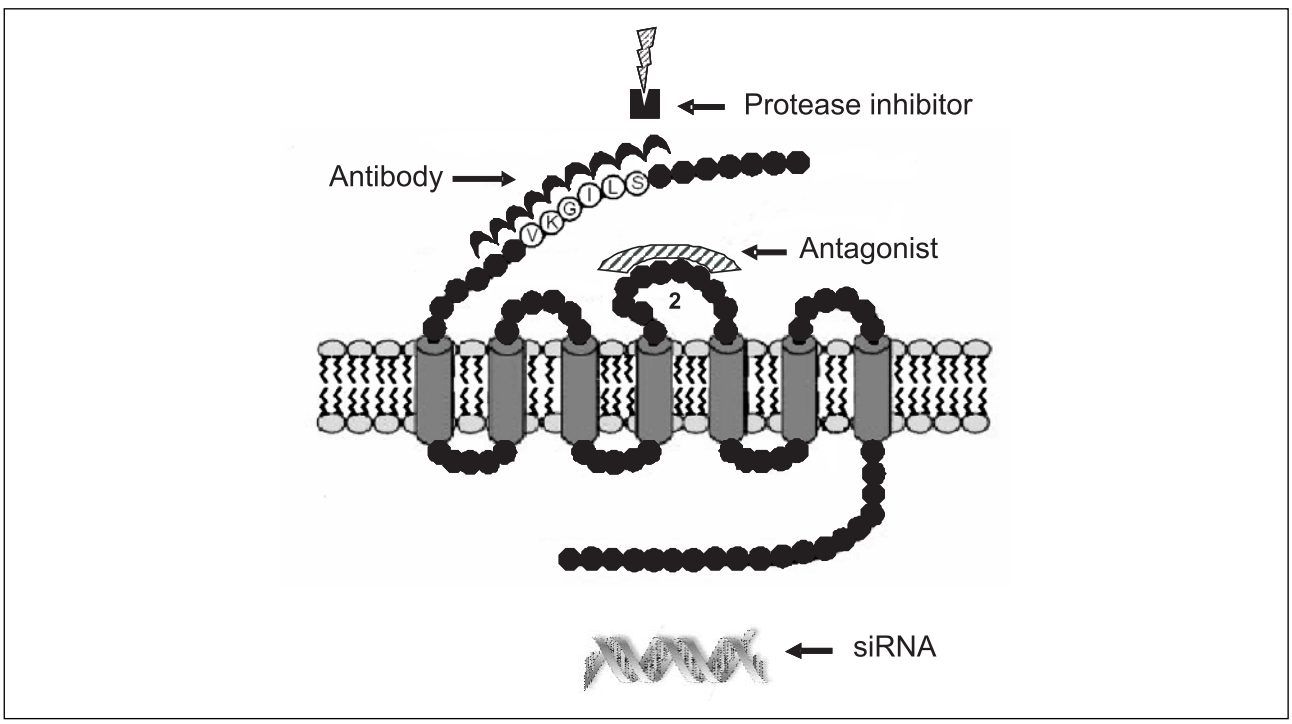


Fig. 3. Potential intervention sites to prevent PAR-2 activation. Prevention of activation can be achieved extracellularly by protease inhibition, administration of an antibody spanning the cleavage site or of an antagonist blocking the ligand-docking site on loop 2 of the receptor. PAR-2 gene silencing can be achieved by delivery of siRNA.

## Conclusions and future directions

The various studies described in this review suggest that PAR-2 could represent an important new therapeutic target in RA. There is a need to improve the management of this condition as current treatment is suboptimal. Although anticytokine (biologic) therapy has been a significant advance, the fact that such agents are usually co-administered with conventional disease-modifying antirheumatic drugs (DMARDs), such as methotrexate, indicates suboptimal efficacy. In addition, not all patients respond to such therapy, these agents cannot be administered orally and they can have significant side effects.

There are a number of possible approaches to preventing PAR-2 activation. A genomic approach involving the use of siRNA is possible (16), although this would require the administration of a viral or other type of vector to optimize efficacy. Inhibition of serine proteases seems an obvious target, and although tryptase appears to be a likely candidate, it is clear that other proteases can activate PAR-2, which limits the effectiveness of this approach.

Antagonism of PAR-2 with a low-molecular-weight, orally active compound such as ENMD-1068 would be valuable, but currently such agents are of low potency. Consequently, the alternative approach of preventing proteolytic activation of PAR-2 using antibodies targeted at the epitope spanning the serine protease cleavage site has been adopted. This approach, known to be successful at inhibiting PAR-2 activation (16), has resulted in the patenting of monoclonal antibodies directed to this epitope on PAR-2 (59). However, the problem of delivery of such antibodies remains, as these proteins cannot be administered orally, and recent adverse events associated with the use of humanized antibodies have given cause for concern with this approach.

Other clinical strategies, such as viral gene delivery of siRNA, could be utilized, as proven in an animal model of arthritis (16), but concerns with viral delivery in humans and the fact that the early promise of gene therapy has not been realized have dampened the initial enthusiasm for this approach. Targeting downstream intermediates in the PAR-2 cascade may constitute an alternative strategy. For example, PAR-2 activation in the synovium leads to enhanced expression of CCL19 (48), a chemokine providing a potent migration and maturation signal for dendritic cells (60). Inhibiting this chemokine could prove to be an effective approach. Alternatively, the development of pharmacological agents that inhibit intermediates in the PAR-2 signaling pathway, such as drugs targeting IKK2 (61), may prove to be effective, particularly if these are low-molecular-weight, orally active compounds. Ultimately, only clinical studies in RA patients will determine the therapeutic value of PAR-2 inhibition in the treatment of arthritis and perhaps other chronic inflammatory diseases.

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