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**Review Article**

# FX06 (fibrin-derived peptide Bβ15-42) – a potential candidate for myocardial reperfusion therapy

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**Abstract**

FX06 (fibrin derived peptide Bβ15-42) is a promising new drug candidate indicated for the prevention of reperfusion injury in patients undergoing primary percutaneous intervention (PCI) for acute myocardial infarction (AMI). This novel peptide has been shown to prevent myocardial reperfusion injury in a number of acute and chronic animal models for this disease when given as a single bolus injection at the time of reperfusion. FX06 has a novel mechanism of action: it is a competitive inhibitor of the binding of fibrin E1 fragments to vascular endothelial (VE)-cadherin. Through this inhibition, it potently blocks the transmigration of inflammatory leukocytes through the endothelial barrier and prevents the downstream release of tissue-damaging mediators. FX06 has proven safe in acute and subchronic toxicological studies and recently entered clinical development.

## Introduction

Cardiovascular disease remains the number one cause of mortality in the industrialized world. In 2003, 13.2 million people in the U.S.A. alone had coronary artery disease (CAD), and 7.2 million had suffered some form of myocardial infarction. The incidence of new cases is 1.2 million annually, of which 42% eventually die from the condition. Coronary infarction claimed 480,000 lives in the U.S.A. in 2001. In the E.U., based on the latest available data, 310,000 patients die annually from coronary heart disease (1)

Today, revascularization with the use of a balloon catheter (percutaneous coronary intervention, PCI) has become the gold standard of treatment for acute myocardial infarction (AMI). The majority of patients also have stent implantation. Drug-eluting stents hold great promise for the prevention of restenosis (2). An unsolved problem is the development of reperfusion injury after these procedures, which is due to the sudden re-initiation of blood flow and the occurrence of local acute inflammation leading to irreversible endothelial and myocardial damage (3). The benefit of reperfusion is thereby significantly reduced.

A description of the phenomenon of reperfusion injury appeared in the literature for the first time in 1935 (4). Even though there was quite a bit of debate about the relevance of lesion development in myocardial infarct patients, it is now widely accepted that a major component of the final infarct size is indeed caused by reperfusion injury (5). There is no doubt that infarct size needs to be limited to the maximum possible extent, because in patients with AMI the prognosis is dependent on the amount of myocardial damage partly arising from ischemia/reperfusion injury (6, 7).

Over the past 30 years, a large number of experimental interventions (both pharmacological and nonpharmacological) have been reported to protect the ischemic myocardium in experimental animal models of ischemia/reperfusion. However, the only finding that translated into clinical practice is related to the establishment of perfusion at the earliest possible time point. In 1971, it was proposed that it should be possible to influence the extent and severity of tissue damage after coronary occlusion by therapeutic strategies, since the amount of tissue lost is not completely predetermined at the onset of ischemia (8). In 1974, Braunwald and

Maroko proposed that cardioprotective therapies be tested in clinical trials (9).

The problems encountered since that time with a large number of therapeutic approaches have been critically appraised by Bolli *et al.* (10). According to their evaluation, major contributors to the clinical failures were lack of reproducible basic research findings, the use of inadequate animal models and poor design of clinical trials.

FX06, a peptide derived from the fibrin sequence, has antiinflammatory properties and provides a new mechanistic approach to the prevention of ischemia/reperfusion injury. It has shown consistent and pronounced activity in a number of animal models predictive of inflammation, including acute and chronic models of myocardial ischemia/reperfusion (11). Clinical development of this product was recently initiated.

## Reperfusion injury and inflammation

The underlying biological mechanisms leading to ischemia/reperfusion injury are quite complex. Numerous experimental investigations have provided evidence that multiple effector systems are activated in a staged process. An early component is the formation of activated oxygen species (12). Calcium overload (13) also plays an important role, as does activation of the complement system (14). A central feature of the inflammatory process as a major culprit in ischemia/reperfusion injury is the migration of leukocytes from the circulation, across the endothelium and the basement membrane, and into the affected myocardial tissue. This extravasation (also called diapedesis) is initiated by chemokines, which are present at the site of injury on endothelial cells and produce a chemotactic gradient (15). This gradient attracts leukocytes, which are then tethered and start rolling on the endothelial surface by the interaction of selectins with their carbohydrate counterligands. Subsequent firm adhesion to the endothelial layer is thought to be caused by binding of leukocyte integrins with their ligands, the immunoglobulin-like intercellular adhesion molecules (*e.g.*, VCAM and ICAM) (16) (Fig. 1).

Finally, leukocytes have to open the multilayered molecular zipper of molecular interactions forming the junction between neighboring endothelial cells via a tightly coordinated multistep process. Molecules configuring this zipper include junctional adhesion molecules (JAMs), platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31), CD99 and VE-cadherin (CD144). Each of these molecules participates in stabilizing the junction by homophilic adhesion with neighboring endothelial cells. JAMs, PECAM-1 and CD99 contribute to leukocyte transmigration by building homotypic and/or heterotypic interactions with their partners on leukocytes (17) (Fig. 2).

It has recently been shown that an interaction of fibrin E1 fragments with VE-cadherin plays a crucial role in opening the endothelial junction and engaging leukocytes to the intercellular space between endothelial cells. The binding site of fibrin and fibrin E1 fragments has been mapped to the β-chain *N*-terminus beginning with amino acid 15 (18). Moreover, fibrin E1 fragments have a binding site for CD11c/CD18, which is located in the A α-chain (19). This dual binding affinity enables fibrin E1 fragments

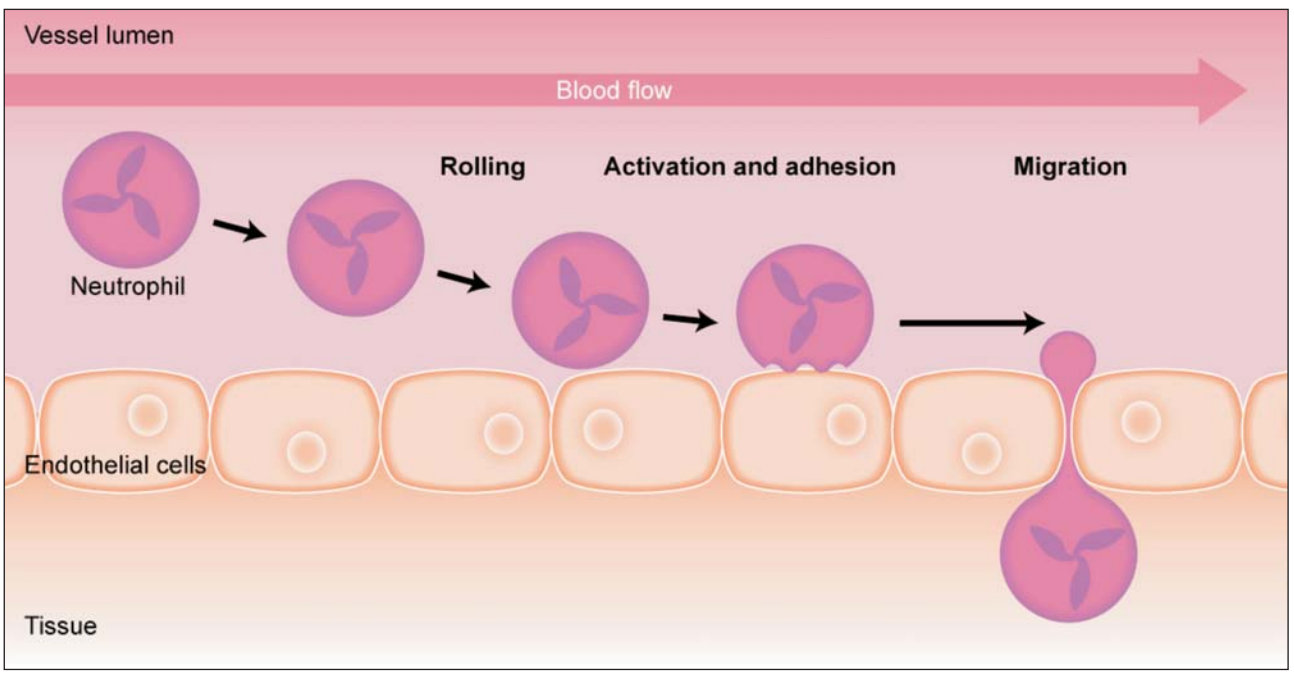


Fig. 1. Consensus mechanism of leukocyte adhesion and transmigration. Tethering and rolling is induced through the interaction of selectins with their carbohydrate receptors. Firm adhesion is orchestrated by binding of leukocyte integrins with their cognate receptors. Finally, migration into tissue follows a highly orchestrated process, in which a multilayered molecular zipper of molecular interactions between neighboring endothelial cells is opened up.

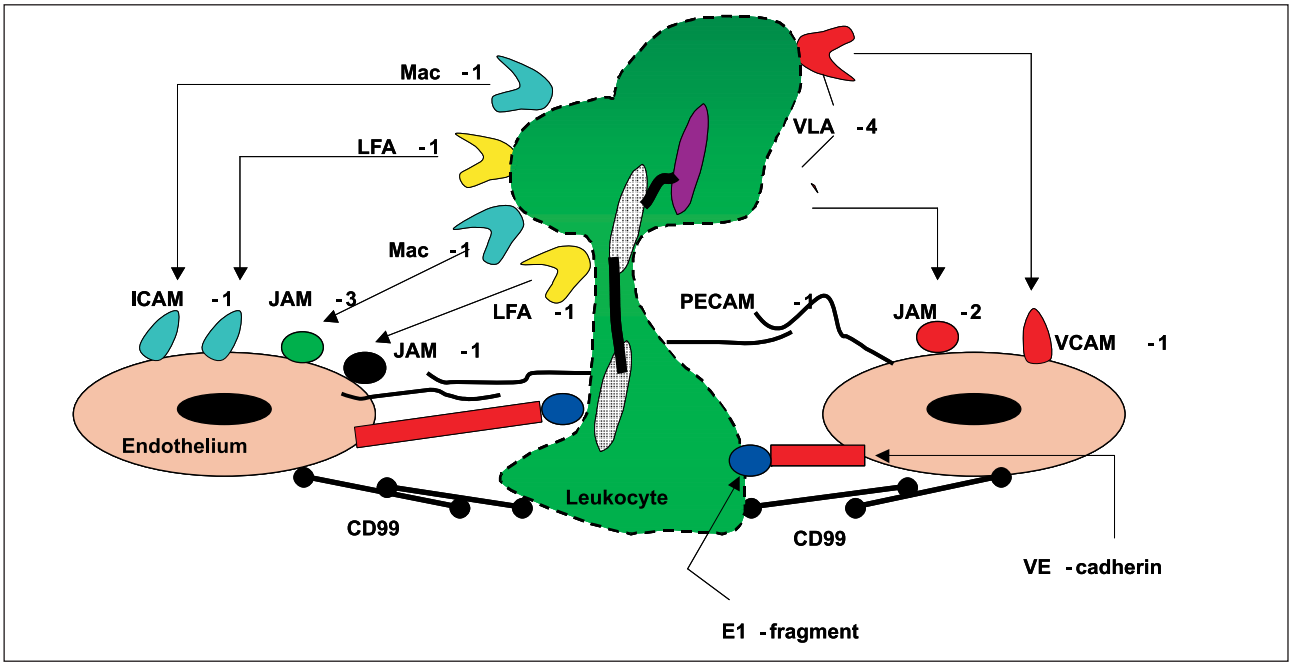


Fig. 2. Scheme illustrating leukocyte diapedesis. To leave postcapillary venules and reach tissues, circulating leukocytes undergo a tightly coordinated multistep process. At sites of endothelial activation, leukocytes tether, roll, become activated and adhere to endothelial surfaces. Up to this point, the process is fully reversible and leukocytes can "fall back" into the bloodstream. The final step, transmigration through endothelial junctions, is thought to be irreversible. Leukocytes must cross the multilayered molecular zipper of interendothelial junctions. Molecules configuring this zipper include, *e.g.*, junctional adhesion molecules (JAMs), platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31), CD99 and VE-cadherin (CD144). Each of these molecules participates in zipper formation by homophilic adhesion with neighboring endothelial cells. JAMs, PECAM-1 and CD99 contribute to leukocyte transmigration by building homophilic and/or heterotypic adhesion to leukocytes. For VE-cadherin, we recently described a novel indirect mechanism by which this molecule is able to interact with inflammatory cells, namely, the E1 fragments of fibrin. These fragments build a bridge between VE-cadherin and leukocytes, thereby directing cells into tissues. (Reprinted with permission from Zacharowski *et al.* J Mol Med, Copyright 2006 by Springer.)

to act as a bridge between inflammatory white cells and the endothelial junction. This bridging effect can be expected to facilitate the movement from the bloodstream into tissues.

## Therapeutic approaches targeting the inflammatory process

Over the last three decades, a number of groups have attempted to develop a therapy for ischemia/reperfusion injury by interfering with the receptor interactions that control leukocyte rolling and endothelial attachment. Although promising experimental data have been obtained in animal studies, the drugs have generally been disappointing in clinical trials. These approaches were recently expertly reviewed (20, 21). As an example, it should be mentioned that the HALT-MI trial with the monoclonal antibody Hu23F2G (LeukArrest), targeting the CD11/CD18 receptor, failed to show a reduction in infarct size in 420 patients undergoing primary angioplasty (22).

These failures may be attributable to the redundant nature of these receptor interactions, where blockade of one receptor interaction still leaves the remaining interactions to promote the overall process (23). For this reason, a promising therapeutic approach needs to focus on an irreversible step, which also constitutes a bottleneck in the overall inflammatory process. There is recent evidence that interfering with the interaction of fibrin E1 fragments with VE-cadherin may be such a promising approach.

## Fibrin/fibrinogen and inflammation

There is increasing evidence that the plasma protein fibrinogen, which is constitutively expressed in the liver, plays a major role in the inflammatory process. Several studies have proposed a role for fibrinogen in the stimulation of inflammatory mediators such as IL-1β (24), IL-8 (25), macrophage inflammatory proteins and monocyte chemoattractant protein-1 (MCP-1) (26). The Framingham study, where 1,315 participants who were free of cardiovascular disease were followed for 12 years, confirmed blood levels of fibrinogen as an independent risk factor for cardiovascular disease, of comparable importance to blood pressure, hematocrit, obesity, cigarette smoking and diabetes. In addition, fibrinogen values were significantly related to these risk factors, indicating that fibrinogen should be included in the profile of cardiovascular risk factors (27).

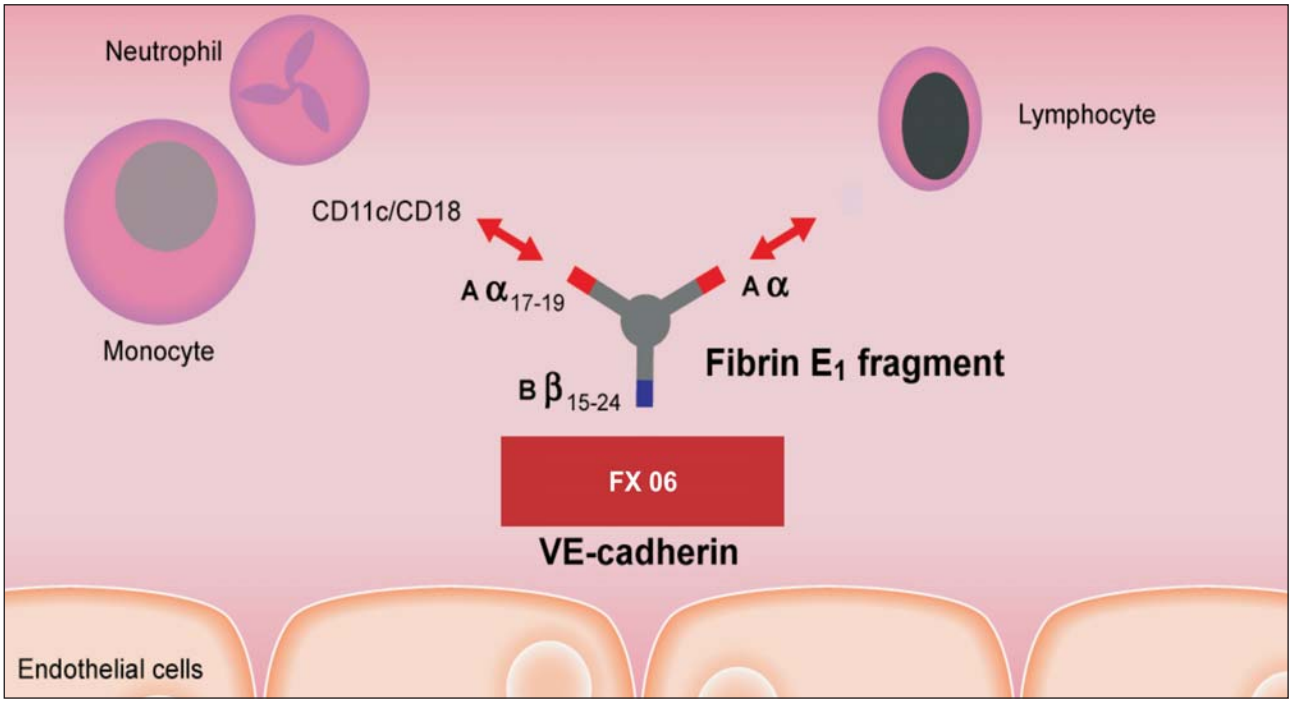


Fig. 3. Binding sites on E1 fragments. E1 fragments are composed of the *N*-terminal segments of fibrin (39). These fragments carry a binding site for VE-cadherin localized at the *N*-terminus of the β-chain and a binding site for CD11c localized at the *N*-terminus of the alpha-chain (40). By building a bridge between inflammatory cells and endothelium, E1 fragments induce transmigration.

This finding was confirmed by Koehler *et al*., who reported that plasma D-dimer levels were independently associated with the presence of CAD in patients with stable angina pectoris. Moreover, plasma concentrations of D-dimers and fibrinogen were independently correlated to the severity of stable angina after myocardial infarction in patients with atherosclerosis (28, 29). Another group determined the role of D-dimer in 230 patients with unstable angina pectoris on presentation to the emergency department. D-dimer levels were significantly correlated to cardiac risk factors and to the length of stay in the hospital (30). Finally, levels of soluble fibrin monomers are considered a predictor of mortality in patients with myocardial infarction (31).

A recent study evaluated the contribution of hematological factors and chronic inflammation to the development of myocardial infarction at a young age (< 45 years old). Plasma levels of soluble fibrin and C-reactive protein (CRP) were significantly higher in patients than in controls. On multivariate regression analysis, soluble fibrin was the strongest predictor of myocardial infarction (32). These data provide strong evidence for the role of fibrin formation and degradation as important events in the development, progression and prognosis of cardiovascular disease.

Binding sites for various integrins or intercellular adhesion molecule-1 (ICAM-1) have been described to reside on fibrin D fragments (33-38). Fibrin-derived E1 fragments on the other hand have epitopes that bind to VE-cadherin (18) and CD11c (19). Proinflammatory activities of fibrinogen and fibrin-derived degradation products have been investigated in our laboratories. An important finding was the stimulation of leukocyte transmigration through endothelial cell monolayers by fibrin E1 fragments *in vitro*. This function of the E1 fragment is based on its ability to bind to the endothelial cell adhesion molecule VE-cadherin (Fig. 3). This interaction is mediated by the *N*-terminal amino acids of the fibrin β-chain, covering amino acids Bβ15-42. Using a small peptide with exactly this sequence (peptide Bβ15-42), known as FX06, we were able to block E1 fragment-induced transmigration *in vitro* (11).

## Chemistry

Fibrin formation is initiated by the cleavage of the fibrinopeptides A and B from fibrinogen by thrombin. The resultant fibrin monomer can polymerize to nonstabilized polymers, which can subsequently be cross-linked in the presence of calcium and factor XIII (activated by thrombin) to form stabilized fibrin. The glutamyl-lysyl cross-links form rapidly between the γ-chains to form γ-dimers, while the α-chains cross-link more slowly to form α-chain polymers with a molecular weight in excess of 400,000 (39).

Fibrinolysis is initiated by cleavage of fibrin by plasmin to form D and E1 fragments. While D fragments recombine to form a D-dimer, which can be measured in serum, E1 fragments are rapidly degraded further, with the formation of E2 and E3 fragments. The initial cleavage from E1 to E2 fragments gives rise to the peptide Bβ15-42, which can be measured in serum as well (40).

FX06 (fibrin-derived peptide Bβ15-42) is composed of all-natural amino acids and has the following amino acid sequence: **H-Gly-His-Arg-Pro-Leu-Asp-Lys-Lys-Arg-Glu-Glu-Ala-Pro-Ser-Leu-Arg-Pro-Ala-Pro-Pro-Pro-Ile-Ser-Gly-Gly-Gly-Tyr-Arg-OH**

or

**GHRPLDKKREEAPSLRPAPPPISGGGYR** in the one-letter annotation.

It can be conveniently prepared by step-wise solid-phase synthesis using standard Fmoc chemistry starting with H-Arg(Pbf)-Wang resin. Simple purification is possible via reverse-phase chromatography. Batches of up to 150 g of peptide have been manufactured under cGMP conditions. The peptide is very soluble in buffered solution and remains stable in solution for at least 1 year. For clinical development it is formulated as a ready-made sterile solution in phosphate buffer and physiological saline.

## Pharmacological actions

The fibrin-derived peptide FX06 competes with *N*-terminal disulfide knot-II (NDSK-II, fibrin E1 fragment analogue) for binding to VE-cadherin, thereby preventing the transmigration of leukocytes across endothelial cell monolayers. To analyze its pharmacodynamic activity in more detail, the affinity of FX06 for VE-cadherin —the proposed receptor molecule of FX06— was analyzed using an *in vitro* ELISA assay, in which recombinant human VE-cadherin was immobilized onto ELISA plates. The binding of FX06 was concentration-dependent. A control peptide with a random amino acid sequence did not bind to the immobilized VE-cadherin.

In addition, it has been demonstrated that NDSK-II competes with FX06 for binding to immobilized human VE-cadherin. It was therefore established that FX06 is a competitive antagonist of the binding of NDSK-II, a proinflammatory analogue of fibrin E1 fragments formed during fibrin formation and degradation (11).

The fibrin fragment NDSK-II (Aα17-51, Bβ15-118, γ1-78), structurally almost identical to the naturally occurring fibrin E1 fragment (Aα17-78, Bβ15-122, γ1-62), induces leukocyte transmigration through endothelial cell layers in cell culture. This effect is mediated through binding to VE-cadherin. The effect of FX06 on NDSK-II induced transmigration of peripheral blood mononuclear cells (PBMCs) was analyzed *in vitro* in a cell migration assay. It was shown that NDSK-II-induces transmigration of lymphocytes, monocytes and neutrophils across endothelial monolayers in a concentration-dependent and saturable fashion. Using VE-cadherin-transfected ECV304 cells and PECAM-1 transfectants (negative controls), the results confirmed that the proinflammatory effect of NDSK-II depends on VE-cadherin expression. Both the adhesion and transmigration of PBMCs across endothelial cell layers and ECV304 layers are blocked by FX06 *in vitro* (11).

The effects of FX06 on infarct size have been investigated in myocardial ischemia/reperfusion models in rats, mice and domestic pigs. These studies were performed in order to investigate whether FX06 elicits any therapeutic effect during myocardial ischemia/reperfusion *in vivo*, where inflammation plays a major role (11). Intravenous administration of the peptide reduced the infarct size following regional myocardial ischemia (25 min) and reperfusion (2 h) in anesthetized rats. When compared to vehicle controls, infusion of FX06 at 0.1, 0.3, 0.9 or 2.4 mg/kg caused a reduction in infarct size of 3%, 7%, 29% and 40%, respectively. The effect became statistically significant at 2.4 mg/kg. The peptide had no effect on heart rate or mean arterial blood pressure in this model.

To determine if the observed effects on infarct size resulted from an antiinflammatory effect of the peptide, H&E-stained hearts from the acute rat study were evaluated by light microscopy of tissue slices. In control peptide-treated heart sections, numerous neutrophils and mononuclear cells were found in the perivascular area, as well as a diffuse distribution pattern within areas of infarction and also surrounding the infarction, which were clearly reduced in animals treated with peptide Bβ15-42. There was also a correlation between the number of inflammatory cells present and infarct size (% area at risk). This analysis clearly demonstrates that leukocyte infiltration into tissues is also inhibited by treatment with the peptide *in vivo.*

In a porcine model of cardiac ischemia/reperfusion injury, the peptide was given as an i.v. bolus injection of 2.4 mg/kg at the time of reperfusion. Anesthetized young domestic pigs were subjected to 1 h of cardiac ischemia induced by ligation of the left anterior descending artery (LAD), followed by 3 h of reperfusion. Ischemic preconditioning was used as a positive control and consisted of brief ligation of the LAD for 15 min and re-opening of the coronary artery prior to the study procedure. Myocardial damage was measured using Evan's blue to determine the area at risk and 1% triphenyltetrazolium chloride (TTC, 1.0% w/v in PBS) was used to determine infarcted tissue. The final infarct size was 65% of the area at risk for saline controls, 41% for the FX06-treated group and 36% for the preconditioned group, respectively. The difference compared to controls was statistically significant for both treatment groups, while there was no difference between the treated groups (46).

In order to evaluate if the beneficial effect of FX06 on infarct size is maintained over a prolonged period of time following a single bolus injection at the time of reperfusion, the drug was administered in a chronic rat model of ischemia/reperfusion. Ischemia was produced by LAD occlusion for 25 min, followed by 7 weeks of reperfusion. In animals treated with FX06 (single bolus of 2.4 mg/kg), infarct size was reduced by 34% compared to controls. Histological analysis of heart sections revealed that scar formation and myocardial remodeling were significantly reduced in FX06-treated rats when compared to controls (11).

## Toxicology and clinical studies

In pharmacological safety studies, FX06 had no effect on hemodynamic and respiratory parameters, nor on

coagulation parameters. In addition, no effect was seen on the electrocardiogram (EKG), especially on Q-T and Q-$T_c$ intervals. The drug was extremely well tolerated in acute and subchronic toxicology studies in three species (mice, rats and dogs), even at very high doses.

More recently, a first-in-man trial confirmed the extremely benign safety profile of FX06. Healthy subjects were given up to 1350 mg as two divided boluses over 10 min. No treatment-related adverse events were observed (manuscript in preparation). Pharmacokinetic evaluation showed a half-life in man of approximately 17 min. As expected with a peptide of this chain length, it is predominantly cleared by renal filtration (47).

A first clinical trial in patients with AMI undergoing PCI has recently been started. In this trial, the effect of 400 mg of peptide given at the time of reperfusion on myocardial salvage after PCI will be measured. The drug is given on the background of standard therapy. Results are expected within a year.

## Other current therapeutic approaches for reperfusion injury

Several other approaches are currently under clinical investigation. While some of these approaches also target aspects of inflammation, others attempt to inhibit the apoptotic death of myocardial cells, a hallmark of reperfusion injury. These latter approaches may potentially act in synergy with FX06.

Since the complement system is known to be activated during reperfusion injury, resulting in the formation of anaphylotoxins C3a, C4a and C5a, as well as the membrane attack complex, Alexion Pharmaceuticals has been developing a monoclonal antibody against C5, known as pexelizumab. This antibody binds to the C5 convertase site and prevents C5 cleavage (41). It has recently been reported that this drug failed to show a statistically significant benefit in the 5000+-patient APEX-AMI trial. Equivocal results were seen in the PRIMO-CABG trials in bypass surgery.

ParinGenix is developing PGX-100, a 2-*O*,3-*O*-partially desulfated heparin. It is claimed that this molecule has lost 97% of the anticoagulant properties of heparin, but retains the full antiinflammatory activity. This product is in phase I/II clinical development (42).

TGI-100-115 is under development by TargeGen. It is a subspecies-specific inhibitor of phosphatidylinositol 3-kinase and is claimed to prevent vascular endothelial growth factor (VEGF)-induced edema, thereby preventing microvascular obstruction and tissue damage (43).

INO-1001 is an inhibitor of poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme that catalyzes the synthesis of long branching (ADP-ribose) polymers from $NAD^+$. This enzyme is an important regulator of apoptotic cell death. Its inhibition is hypothesized to protect myocytes in the area at risk during reperfusion (44).

A similar approach is being followed by KAI Pharmaceuticals and Daiichi Sankyo, which are co-developing KAI-9803 (CS-9803), a peptide inhibitor of the translocation of protein kinase C δ (PKCδ) to the mitochondrial membrane. This inhibition stops the mitochondrial apoptotic pathway and is also potentially protective for myocardial tissue (45).

All of these compounds are in early clinical development.

## Summary and perspectives

The clinical prognosis for patients with AMI has significantly improved with the technical progress in the PCI procedure. Most significantly, early referral to experienced centers with catheter laboratories and shortening the period to intervention have resulted in reduced mortality and late complications. However, the clinical success of PCI is still hampered by the development of reperfusion injury caused by acute inflammatory reactions. Despite three decades of intense preclinical and clinical investigation, no product for the prevention of reperfusion injury has yet reached the market. Novel approaches which target critical steps in the occurrence and progression of reperfusion injury are desperately needed. Several promising drug candidates are currently under early clinical investigation based on a rational understanding of the underlying pathophysiology of the disease. Prominent among these drug candidates is FX06. An impressive body of experimental evidence supports its clinical development in this indication.

Currently ongoing proof-of-concept clinical trials with these new drugs will show within 12-18 months if there is potential for further improvement in the treatment of patients with myocardial infarction.

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