Title: INHIBITION OF RENAL ISCHEMIA REPERFUSION INJURY BY SYNTHETIC Oligopeptides

Abstract: The invention relates to the field of medicine. In particular, it relates to the therapeutic use of synthetic oligopeptides capable of inducing cell regeneration, for instance in the treatment of ischemic renal injury. Provided is the use of the peptide AQGV for the preparation of a medicament for the treatment of a subject in need of cell regeneration. Also provided is a method for the treatment of a subject in need of cell regeneration, comprising administering to said subject a therapeutically effective dose of the peptide AQGV together with a pharmaceutically acceptable carrier.
Title: Inhibition of renal ischemia reperfusion injury by synthetic oligopeptides.

The invention relates to the field of medicine. In particular, it relates to the therapeutic use of synthetic oligopeptides capable of inducing cell regeneration, for instance in the treatment of ischemic renal injury.

Ischemic acute renal failure (ARF) is the most common cause of intrinsic ARF in adults [1, 2]. In native kidneys, it is associated with an overall mortality rate of up to 50% [3]. Despite advances in preventative strategies and developments in dialysis treatment, this disease continues to be associated with significant morbidity and mortality [4]. In addition, ischemia reperfusion (IfR) injury after kidney transplantation is associated with primary non function [5] and decreased long-term graft survival [6, 7].

Inflammation plays a major role in the pathophysiology of renal ischemic injury [8]. The initial ischemic injury results in up-regulation of adhesion molecules on activated endothelium and release of inflammatory mediators, including cytokines, reactive oxygen species (ROS) and eicosanoids. Leukocytes, recruited by chemokines and pro-inflammatory cytokines, like TNF-α, potentiate injury by further generating more ROS and eicosanoids, enhancing inflammation.

Recently, we have reported that oligopeptides related to the primary structure of the β-chain of human chorionic gonadotropin (hCG) are capable of inhibiting the acute inflammatory response [9, 10].

hCG is a hormone produced during pregnancy by placental trophoblasts [11], but is also produced by the pituitary gland and leukocytes in non-pregnant females and males [12, 13]. It consists of an α- and β-chain. The α-chain is similar to that of other glycoprotein hormones, while the β-chain accounts for the unique properties of hCG.
In human pregnancy urine and in commercial hCG preparations (c-hCG) hCG occurs in a variety of forms, including breakdown oligopeptide products. During pregnancy, the urine contains increasing proportions of nicked hCG and hCG β-core fragments [14]. Nicked hCG has peptide bond cleavages in loop 2 of the β-chain between residues 44 and 52, whereas hCG β-core completely lacks the β-chain loop 2, which consists of amino acid residues 41-54.

We have previously shown that the 400 - 2000 Dalton fraction of pregnancy urine, but not of normal female or male urine, is able to inhibit the development of diabetes in NOD mice, whereas fractions greater than 2000 Dalton, including hCG, did not have this activity [15]. Furthermore, the synthetic hexapeptide VLPALP, which is part of the primary structure of the β-chain loop 2, was found to reduce mortality in a murine model for lipopolysaccharide (LPS) induced systemic inflammatory response syndrome [9].

Based on these findings, and known preferential cleavage sites of the hCG β-chain loop 2 [14, 16-19], we selected six different synthetic oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP and VLPALPQ) as well as four alanine variants of LQGV (AQG, AQGV, LAG and LAQV), and tested these in a murine model for their renal I/R injury reducing capacity.

To this end, ten different oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, AQG, AQGV, LAG or LAQV) were administered at a dosage of 5 mg/kg BW 1 minute before induction of ischemia and 1 minute before reperfusion. The mice were sacrificed at 6, 24 and 72 hours post-reperfusion. Survival, kidney function, inflammatory cells, serum cytokine levels and mRNA levels for genes of interest were determined.

Survival at 72 hours post reperfusion was significantly higher in mice treated with either MTRV, LQG, VLPALPQ or AQGV as compared to placebo treated mice. Some oligopeptides were more effective than others. AQGV
completely prevented mortality and best preserved kidney function. Since AQGV showed the most robust protection against renal I/R injury, it was tested in a dose-escalating study in a range of 0.3 to 30 mg/kg. A survival gain was observed with all doses. Improvement of kidney function was observed from 1 mg/kg. Highest survival and best preserved kidney function was observed at 3 and 10 mg/kg. No toxicity was found at all doses tested.

Upon treatment with AQGV the levels of E-selectin mRNA in kidneys were significantly decreased at 6 hours post-reperfusion. Immunohistochemistry of kidneys of AQGV-treated mice revealed a significantly lower influx of neutrophils both at 24 and 72 hours. Serum levels of TNF-α, INF-γ, IL-6 and IL-10 were significantly decreased in the AQGV treated mice at 24 hours post reperfusion. Renal tubular epithelial cell proliferation was significantly increased in AQGV-treated mice at 24 hrs post-reperfusion.

 Accordingly, in one embodiment the invention provides the use of AQGV for the preparation of a medicament for the treatment of a subject in need of cell regeneration, preferably epithelial or renal tubular cell generation. Provided is the use of AQGV for the manufacture of a medicament for the treatment of a condition or disorder that would benefit from cell regeneration. Exemplary diseases include ischemic renal injury and ischemia reperfusion (I/R) injury, e.g. as can be observed after kidney transplantation.

Also provided are methods for enhancing survival and preservation of kidney function, comprising the administration of a therapeutically effective dose of AQGV. Suitable doses range from 0.001 mg to 1000 mg/kg of body weight, preferably 0.1 mg to 500 mg/kg of body weight. Good results can be obtained with at least 1 to about 50 mg/kg of body weight, for example 3, 5 or 30 mg/kg.

Provided herein is the therapeutic use of AQGV for treating unwanted conditions or symptoms that are associated with increased E-selection expression. E-selectin, also known as CD62E, is a cell adhesion molecule
expressed only on endothelial cells activated by cytokines. Like other selectins, it plays an important part in inflammation. E-selectin recognises and binds to sialylated carbohydrates present on the surface proteins of certain leukocytes. These carbohydrates include members of the Lewis X and Lewis A families found on monocytes, granulocytes, and T-lymphocytes. During inflammation, E-selectin plays an important part in recruiting leukocytes to the site of injury. The local release of cytokines IL-1 and TNF by damaged cells induces the expression of E-selectin on endothelial cells of nearby blood vessels. Leukocytes in the blood expressing the correct ligand will bind with low affinity to E-selectin, causing the leukocytes to "roll" along the internal surface of the blood vessel as temporary interactions are made and broken. As the inflammatory response progresses, chemokines released by injured tissue enter the blood vessels and activate the rolling leukocytes, which are now able to tightly bind to the endothelial surface and begin making their way into the tissue.

Legends to the Figures

Figure 1. Renal function as reflected by blood urea nitrogen level (BUN; mean ± SEM). Pre-operative values and 24 and 72 hours post reperfusion values in the different oligopeptide treated groups were compared to PBS-treated controls. Treatment with MTRV and AQGV significantly reduced renal function loss after ischemia reperfusion (I/R) injury. Treatment with LQG reduced the injury only at 24 hours, not at 72 hours. Treatment with VLPALPQ and LAGV showed significantly reduced renal function loss at 72 hours. * = p<0.05 or# = pO.Ol (n = 10 per group).

Figure 2. Renal function as reflected by blood urea nitrogen (BUN; mean ± SEM). Values are shown pre-operative, and after 24 and 72 hours in groups treated with AQGV in a dose escalation study (0.3 - 30 mg/kg), and compared to a PBS-treated control group. Treatment with AQGV in a dose from 1
mg/kg up to 30 mg/kg significantly reduced renal function loss after renal I/R injury. The dose of 3 mg/kg was the most potent. * = p<0.05 and # = p<0.01 (n = 10 per group).

Figure 3. Renal neutrophil influx as assessed by immunohistochemical staining.
AQGV treatment reduced neutrophil infiltration after 25 minutes of renal warm ischemia as assessed at 24 and 72 hours post-reperfusion. Data are expressed in a semi-quantitative way as described in the Material and Methods section. * = p<0.05 (n = 10 per group).

Figure 4. AQGV treatment significantly enhanced cellular proliferation at 24 hours after renal I/R injury. Although a higher trend of proliferation was seen at 72 hours as well, no statistically significant difference was found. Proliferation was assessed by immunohistochemical staining for the proliferation marker Ki-67. Data are expressed as in a semi-quantitative way as described in the Material and Methods section. * p<0.05 (n = 10 per group).

Figure 5. Treatment with AQGV reduces serum cytokine levels at 24 hours after renal ischemia reperfusion injury. Cytokine levels are depicted as mean value ± sem. *p<0.05, f p<0.01 (n = 5 per group).

Figure 6. Treatment with AQGV reduces renal E-selectin mRNA levels at 6 hrs after renal ischemia reperfusion injury. Data are presented as mean value ± sem. * p < 0.05 (n = 6 per group).

EXPERIMENTAL SECTION
Materials and Methods
Experimental design. The experimental protocol was approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

Ten different hCG-related oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, AQG, AQGV, LAG and LAGV) were evaluated for their I/R reducing capacity. The effect was compared to mice treated with phosphate-buffered saline (PBS). The study was performed in a double blind manner. 5 mg/kg BW of oligopeptide or PBS in a volume of 0.1 mL was administered intravenously (iv) 1 minute before clamping the kidney, and 5 mg/kg 1 minute before releasing the clamp.

Subsequently a dose-escalating study was performed with AQGV. The AQGV was given in doses of 0.3, 1, 3, 10, and 30 mg/kg in a volume of 0.1 mL and was administered i.v. 1 minute before clamping, and 1 minute before releasing the clamp. Possible toxic side effects were studied by careful observation of control and peptide treated mice for signs of discomfort such as hunched posture, ruffled fur, and diarrhea.

Contra-lateral kidney samples were obtained for further analysis. At 24, and 72 hr post-reperfusion, mice were sacrificed and clamped kidneys were harvested and snap frozen for further analysis. Blood urea nitrogen (BUN) were measured in serum. Infiltrating cells were analysed using immunohistochemistry. In all groups survival was assessed and analyzed by Kaplan-Meier analysis.

In an additional experiment AQGV was given in a dose of 5 mg/kg BW in a volume of 0.1 mL and administered i.v. 1 minute before clamping, and 1 minute before releasing the clamp. At 6 and 24 hr post-reperfusion, mice were sacrificed and blood was obtained for cytokine measurements in serum. From the 6hr post-reperfusion group the clamped kidney was harvested for determination of mRNA expression levels.
Mice. Male C57BL/6JOlaHsd mice of 12-16 weeks of age were obtained from Harlan (Horst, The Netherlands). Mice were kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 h light/12 h dark) and were allowed free access to food (Hope Farms, Woerden, The Netherlands) and water throughout the experiments.

Ischemia model. Mice were anaesthetized by isoflurane inhalation. Anaesthesia was maintained using a mixture of N2O/O2/isoflurane. At the start of the experiments blood was collected by retro-orbital puncture, for determination of pre-operative kidney function. Normal body temperature was maintained by placing the mice on heating pads until recovery from anaesthesia. Following a midline abdominal incision, the left renal pedicle was localized and the renal artery and vein were dissected. An atraumatic microvascular clamp was placed, and the left kidney was occluded during 25 minutes. After inspection for signs of ischemia, the wound was covered with PBS soaked cotton and the animal was covered with a tin foil insulation sheet. After release of the clamp, restoration of blood-flow was inspected visually and a contra-lateral nephrectomy was performed. The excised right kidney was snap frozen and stored at -80°C for further analysis. The abdominal wound was closed in two layers, using 5/0 sutures (B. Braun, Melsungen, Germany), and mice were given 0.5 ml PBS subcutaneously.

Oligopeptides. Selection was based on either the known preferential cleavage sites or known in vivo nick sites of the sequence MTRVLQGVLPALPQ (aa41-54) of loop 2 of the β-subunit of hCG [14, 16-19]. Selected oligopeptides were MTR (aa41-43), MTRV (aa41-44), LQG (aa45-47), AQG and LAG (alanine replaced oligopeptides of LQG), LQGV (aa45-48), AQGV and LAGV (alanine replaced oligopeptide of LQGV), VLPALP (aa48-58), VLPALPQ (aa48-54). Oligopeptides were synthesized (Ansynth BV, Roosendaal, The Netherlands) using the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl-based methodology with a 2-
chlorotriyl chloride resin as the solid support. Oligopeptides were dissolved in PBS at a concentration of 1 mg/ml and stored at -20°C in small aliquots.

Functional measurements. Blood samples were collected by retro-orbital puncture pre-operatively and on time points when animals were sacrificed. Blood Urea Nitrogen (BUN) was measured in serum using the Jaffe method without deproteinization [20].

Immunohistochemistry. Frozen sections (5 µm) were stored at -20°C until use. Primary antibodies that were used were rat-anti-mouse CD4, CD8, CD45, neutrophils, macrophages, CD54 (Serotec). All antibodies were diluted in PBS/5% BSA solution. Slides were air dried and fixed in acetone for 10 min at room temperature (RT), rinsed with PBS and primary antibody was applied for 30 min at RT. The slides were rinsed two times with PBS and incubated with a mixture of goat-anti-rat IgM+IgG (H+L) alkaline phosphate conjugated antibody supplemented with 2% mouse serum for 30 min at RT. After rinsing the slides in Tris-HCl pH8 buffer, enzyme detection was performed using Naphthol AS-MX, New Fuchsine, sodium-nitrite and levamisol mixture in Tris-HCl pH8 as a substrate for 30 min at RT in the dark.

Formalin-fixed-paraffin sections (3 µm) were used for Ki-67 staining. Slides were deparaffinized and rehydrated in descending alcohol solutions, rinsed with PBS and boiled, for antigen retrieval, in a 0.01M sodium citrate solution for 30 min in a microwave-oven. After cooling down and rinsing in PBS, endogenous peroxidase was blocked with a 0.03% H2O2 solution, and rinsed again in PBS. The sections were incubated overnight at 4°C with rat-anti-mouse Ki-67 primary antibody. After rinsing two times with PBS, the slides were incubated for 30 min at RT with rabbit-anti-rat IgG conjugated with HRP secondary antibody. Enzyme detection was performed, after rinsing two times with PBS, using DAB as a substrate for 8 min. All slides were rinsed in tap water, counterstained with haematoxylin and rinsed with tap water again.
Alkaline-phosphatase stained slides were covered with aqueous base mounting medium. Peroxidase-stained slides were dehydrated in increasing concentrations of alcohol, cleared with xylene and a cover slip was put on using Pertex. As a negative control the primary antibody was omitted. Positive cells were counted in 10 high power fields (400X) using a semi quantitative scoring system as follows: 1: no positive cells, 1: 1-10 cells, 2: 11-30 cells, 3: 30-60 cells, 4 > 60 cells.

**Real-time quantitative (RQ)-PCR analysis.** Sections of kidney were homogenized and RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). In total 1 µg of RNA was reverse transcribed and RQ-PCR using an AppliedBiosystems 7700 PCR machine (Foster City, CA, USA) was performed as described previously [21]. In all 6h samples the mRNA transcript levels of TNF-α, IFN-γ, IL-6, IL-10, IL-12, MCP-I, and the adhesion molecules E-selectin and ICAM-I were determined. Transcript levels of these genes were quantified by normalization against ABL. Primers and probes used are available upon request.

**Cytokine measurements.** TNF-α, IFN-γ, IL-6, IL-10, IL-12, and MCP-I were measured using a commercially available cytometric bead array (CBA) (BD Biosciences, San Jose, CA, USA). Measurements with the BD CBA kit were done with a BD FACSArray™ Bioanalyzer (BD Biosciences) and analysis of the data was performed using FCAP Array™ software (BD Biosciences). Assay sensitivity was 2.5 pg/ml.

**Statistical analysis.** Survival data were compared by log-rank analysis. Other data were analysed using ANOVA, followed by a Mann-Whitney -U test. Calculations were performed using SPSS v7.0 for Windows. A p value <0.05 was considered statistically significant.

**Results**
Effect of hCG-related oligopeptide treatment on survival

25 minutes of warm renal ischemia and contra-lateral nephrectomy resulted in a survival of 54% in the control group at 3 days post-reperfusion (table 1). The groups treated with oligopeptides MTR, LQGV, VLPALP, AQQ, LAG and LAGV (5 mg/kg), had survival rates not significantly different from controls. Treatment with LQG led to a significant better survival (90%), while treatment with oligopeptides MTRV, VLPALPQ or AQQV totally prevented mortality.

Table 1. Effect of various hCG-related oligopeptides (5 mg/kg) on the survival at 24 and 72 hours post-reperfusion of mice subjected to 25 minutes of left renal warm ischemia. Mice treated with PBS or hCG-related synthetic oligopeptide were compared by log-rank test (n = 10 per group).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival</th>
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<tbody>
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<td>24h</td>
<td>72h</td>
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<tr>
<td>PBS</td>
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<td>LAGV</td>
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Effect of hCG-related oligopeptide treatment on kidney function

Treatment of mice with oligopeptide MTRV, AQQV or LAGV provided significant (p<0.05) functional protection against renal I/R injury at both 24
and 72 hr, as measured by BUN levels (figure 1). Although treatment with LQG resulted in significantly decreased BUN at 24 hours post-reperfusion (p<0.05), at 72 hours no significant beneficial effect was found. While treatment with VLPALPQ did not cause a significant decrease in BUN at 24 hours, BUN at 72 hours was significantly decreased as compared to the control group (p<0.05). Treatment with AQGV provided the most powerful protection against I/R at both 24 hours (p<0.01) and 72 hours (p<0.01).

Effect of different doses of AQGV (0.3 - 30 mg/kg) on survival

Because AQGV showed the most powerful protection against warm renal I/R, we determined the optimal dose of this oligopeptide in a dose escalating study. Therefore, AQGV was administered in doses ranging from 0.3 to 30 mg/kg, and compared to mice treated with PBS. A survival rate of 60% was seen in the control group (table 2). Although treatment with 0.3, 1 and 30 appeared to result in a survival benefit, no significant difference could be measured (80%, 90%, and 80%, respectively). The doses of 3 and 10 mg/kg totally prevented mortality (p<0.05).

Table 2. Effect of different doses of AQGV on the survival of mice subjected to I/R damage. Survival at 24 and 72 hours post-reperfusion of mice subjected to 25 minutes of left renal warm ischemia and treated with increasing doses of oligopeptide AQGV. AQGV- and PBS-treated mice were compared by log-rank test (n = 10 per group).

<table>
<thead>
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<th>Treatment (mg/kg)</th>
<th>Survival</th>
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<td>PBS</td>
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</table>
Effect of different doses of AQGV (0.3—30 mg/kg) on kidney function

Treatment of mice subjected to renal I/R damage with 1, 3, 10 and 30 mg/kg AQGV resulted in significant reduction of BUN levels at 72 hours (p<0.05). A dose of 3 mg/kg resulted in best preservation of kidney function, especially after 72 hrs (p<0.01). With 0.3 mg/kg no significant reduction was observed (figure 2).

Effects of AQGV on cellular infiltration and proliferation.

To study the mechanism underlying the protective effect of hCG-related oligopeptide treatment on renal I/R injury, we investigated the cellular infiltrate and proliferation in the kidneys of mice treated with 5 mg/kg AQGV by immunohistochemistry. A prominent feature of renal I/R injury is the influx of neutrophils. Our immunohistochemical data show that at both 24 and 72 hr post reperfusion the neutrophil influx was significantly decreased in the AQGV-treated group (p=0.03 and p=0.022, respectively) (figure 3). Additional staining for CD4+, CD8+ cells and macrophages revealed no differences between the two groups (data not shown). Ki-67 staining showed a significantly higher proliferative activity of renal tubular epithelial cells in AQGV-treated mice at 24 hours (figure 4). At 72 hours this difference had disappeared.

Effects of AQGV on serum cytokine levels

Using the bead-array we determined the levels of several serum cytokines at 6 and 24 hr post-reperfusion. MCP-I was below the detection limit in all examined samples. No differences in serum TNF-α, IFN-γ, IL-6, IL-10, and IL-12 levels were observed at 6hrs post-reperfusion. At 24 hrs post-reperfusion the levels for all cytokines decreased upon AQGV treatment, with TNF-α, IL-6, IL-10 (p < 0.05), and IFN-γ(p < 0.01) being significantly lower (Figure 5).
Effects of AQGV on renal mRNA transcript levels

AQGV treatment showed no effect on inflammatory cytokine and ICAM-I mRNA levels 6hrs post-reperfusion. AQGV treatment did result in a significant (p < 0.05) down regulation of renal E-selectin mRNA expression at 6hr post-reperfusion as compared to PBS treated mice (Figure 6).

Concluding Remarks

The present invention is the first to demonstrate that oligopeptides as small as three or four amino acids can significantly reduce mortality seen after severe renal I/R injury and can improve kidney function as measured by BUN levels. Especially AQGV showed superior results in enhancing survival and preservation of kidney function after 25 minutes of renal ischaemia. The dose of 3-10 mg/kg proved to be the most potent with regard to reducing mortality as well as blood nitrogen levels. Furthermore, up to 30 mg/kg, no toxicity was observed. Also in rats, dogs and a human phase I study no harmful side effects of single and repeated AQGV administration were found (data not shown).

Both natural hCG and commercial hCG preparations have been investigated for their role on the immune system, because of their putative immunomodulating role during pregnancy in protecting the fetus from rejection [22]. Our previous work [15] shows that short-term treatment of female NOD mice, with a hCG preparation purified from first trimester pregnancy urine, starting prior to the onset of hyperglycemic symptoms, inhibits the development of type I diabetes. Interestingly, however, the anti-diabetic activity of the used hCG preparation did not reside in the heterodimeric hCG molecule, or its subunits, but in a 400-2000 Dalton fraction. Subsequently, we showed in a model of LPS-induced systemic inflammatory response syndrome in mice that treatment with this low weight molecular fraction was capable of inhibiting the septic shock morbidity as well as mortality [9]. The same beneficial effect was obtained with the synthetic oligopeptide VLPALP, which sequence is part of loop 2 of the β-chain of hCG.
These protective effects are likely due to correcting the dysregulation of the immune balance in these models. During pregnancy, hCG occurs in a variety of forms and breakdown products in serum and urine, including intact hCG, α- and β-subunits, nicked hCG, hCG β-core fragment, and smaller peptide fragments. Both nicked hCG and the β-core subunit consist of a β-chain with a defective loop 2. This loop, consisting of the amino acid residues 41-54, is absent in β-core subunit, and is cleaved in nicked hCG [16-19]. Since the immunomodulatory activity of hCG resided in the low molecular weight fraction, we hypothesised that in vivo liberated breakdown products, such as those originating from the proteolytic cleavage of peptide bonds between amino acid residues 41-54, may have significant biological activity [10]. Based on known preferential cleavage sites [14, 16-19], we tested synthetic oligopeptides MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ and, based on alanine replacement mapping, the LQG and LQGV analogs AQG, LAG, AQGV and LAVG. Of these oligopeptides, MTRV, LQG, VLPALPQ and AQGV appeared able to reduce mortality and decline in kidney function induced by warm renal ischemia-reperfusion injury, AQGV being the most effective (table 1 and figure 1).

Cell migration plays an important role during the initial phase of renal I/R injury. Up-regulation of adhesion molecules on endothelial cells, induced by locally produced pro-inflammatory mediators (a.o. reactive oxygen species (ROS), cytokines), is amongst the first changes observed after renal I/R injury and is central to the pathogenesis of ischemic acute renal failure [8, 23]. Subsequently leukocytes become activated by local pro-inflammatory factors, thereby facilitating adherence to endothelial cells and subsequent renal tissue infiltration [8]. Sequestered neutrophils produce mediators such as ROS that induce parenchymal damage, followed by cytokine production by resident renal cells and infiltrating cells, which promotes further tissue damage [8, 24]. It has been demonstrated that renal mRNA expression of the early adhesion molecule E-selectin peaks within 6 hrs post-reperfusion, with neutrophils...
infiltrating in parallel. E-selectin blockage with the selectin specific ligand sPSGL has been shown to inhibit renal neutrophil infiltration after R/I and to preserve kidney function [25]. In mice treated with AQGV we observed decreased E-selectin mRNA levels 6hrs post-reperfusion and decreased renal neutrophil infiltration at 24 hrs post-reperfusion. Additionally, serum levels of the inflammatory cytokines TNF-α, INF-γ, IL-6, and IL-10 were decreased 24 hrs post-reperfusion upon AQGV treatment. This is indicative of decreased renal injury and fits with the preservation of kidney function we observed.

Our data indicate that AQGV treatment protects against renal I/R injury by interfering with early E-selectin upregulation, thereby reducing neutrophil influx, parenchymal damage and cytokine production. So far it is unclear what the molecular mechanism of action is by which AQGV exerts its effects. It is possible that AQGV mediates its effect by an as yet unidentified receptor. However, based on structural features [26] we expect AQGV to penetrate the cell membrane and to exert its mode of action intra-cellularly by interfering with pathways involved in gene regulation. E-selectin is expressed de novo on endothelial cells after transcriptional induction by pro-inflammatory agents [27]. Whether AQGV inhibits the local production of pro-inflammatory mediators that induce E-selectin or directly interferes with the intracellular signalling cascade involved in activating E-selectin transcription is not clear so far.

Although previous work revealed a pathophysiologic role of the T-cell as mediator of ischemic ARF [28, 29], we did not find a significant difference between the AQGV and placebo treated mice in numbers of CD4+, CD8+ T-cells or macrophages. Our data fit with the observation that RAG-I deficient mice (lacking both T- and B-cells) are not protected from renal I/R injury [30]. Ki-67, a marker for cellular proliferation, is part of a nuclear protein complex expressed in the Gl, S, G2 and M phases of the cell cycle in proliferating cells [31, 32]. Our data show that mice treated with AQGV show significantly
increased numbers of Ki-67 positive renal tubular epithelial cells at 24 hr post-reperfusion, reflecting a regenerative process [33] that is likely facilitated due to a reduction in ongoing inflammation-induced tissue injury.

In conclusion, this invention shows that treatment of mice with 5 mg/kg BW of either one of the hCG-related oligopeptides MTRV, LQG, VLPALPQ or AQGV shortly before and immediately after kidney clamping can significantly reduce mortality and ameliorate kidney injury. Of the various oligopeptides evaluated, AQGV appeared to be the most potent one. The renoprotective effect of AQGV was associated with decreased renal E-selectin transcripts, decreased renal neutrophil infiltration and a reduction of systemic levels of TNF-α, IFN-γ, IL-6 and IL10. This data implies that AQGV interferes with the early renal inflammatory response induced by I/R and as such prevents parenchymal damage and organ dysfunction. These new renoprotective oligopeptides show great promise for the prevention and treatment of ischemia induced acute renal failure.

References


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Claims

1. The use of the peptide AQGV for the preparation of a medicament for the treatment of a subject in need of cell regeneration.

2. Use according to claim 1, wherein the subject is in need of epithelial or renal tubular cell generation.

3. The use of AQGV for the manufacture of a medicament for the treatment of a condition or disorder that would benefit from cell regeneration.

4. Use according to any one of claims 1 to 3, wherein the subject suffers from ischemic renal injury or ischemia reperfusion (I/R) injury, in particular I/R injury after kidney transplantation.

5. A method for enhancing survival and preservation of kidney function in a subject, comprising administering to said subject a therapeutically effective dose of AQGV.

6. A method for the treatment of a subject in need of cell regeneration, comprising administering to said subject a therapeutically effective dose of the peptide AQGV together with a pharmaceutically acceptable carrier.

7. Method according to claim 5 or 6, wherein said subject will undergo or has undergone a kidney transplantation.

8. Use or method according to any one of the above claims, wherein the treatment comprises the administration of a 0.001 mg to 1000 mg peptide per kg of body weight, preferably 0.1 mg to 500 mg/kg of body weight.

9. Use or method according to claim 8, comprising the administration of 1 to about 50 mg/kg of body weight, preferably 3 to 30 mg/kg.
### INTERNATIONAL SEARCH REPORT

<table>
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<td>INV. A61K38/07 A61P13/02</td>
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According to International Patent Classification (IPC) or to both national classification and IPC:

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols):

- A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched:

Electronic data base consulted during the international search (name of data base and, where practical, search terms used):

- EPO-Internal, WPI Data, BIOSIS, EMBASE

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>EP 1 300 418 A (UNIV ERASMUS [NL]) 9 April 2003 (2003-04-09). paragraph [0060]; table. 2</td>
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Further documents are listed in the continuation of Box C.

**T** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention.

**X** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone.

**Y** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

**&** document member of the same patent family.

Date of the actual completion of the international search: 19 November 2008

Date of mailing of the international search report: 01/12/2008

Authorized officer: Greif, Gabriel a
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