Preclinical Study of DNA-Recognized Peptide Pyrrole-Imidazole Polyamide Targeting Human TGF-β1 Promoter for Progressive Renal Diseases in Common Marmoset

Masari Otsuki [1], Noboru Fukuda [1,2]*, Takashi Inoue [3], Takayuki Mineshige [3], Tomoyasu Otsuki [1], Shu Horikoshi [1], Morito Endo [4], Masanori Abe [1]*

[1] Division of Nephrology, Hypertension and Endocrinology, Department of Internal Medicine, Nihon University School of Medicine, Tokyo 173-8610, Japan
[2] Research Center Nihon University, Tokyo 101-0061, Japan
[3] Marmoset Research Department, Central Institute for Experimental Animals, Kanagawa 210-0821, Japan
[4] Faculty of Human Health Science, Hachinohe Gakuin University, Hachinohe, Aomori 031-8588, Japan

Author e-mail addresses:
M. Otsuki: mi.piace.hayden.v@gmail.com
N. Fukuda: fukuda.noboru@nihon-u.ac.jp
T. Inoue: inoue-t@ciea.or.jp
T. Mineshige: mineshige@ciea.or.jp
T. Otsuki: totsuki16@gmail.com
S. Horikoshi: horiko19830823@yahoo.co.jp
M. Endo: mendo@hachinohe-u.ac.jp
M. Abe: abe.masanori@nihon-u.ac.jp

***Correspondence:** Noboru Fukuda and Masanori Abe (Double correspondence), Division of Nephrology, Hypertension and Endocrinology, Department of Medicine, Nihon University School of Medicine, Ooyaguchi-kami 30-1, Itabashi-ku, Tokyo 173-8610, Japan. Phone: 81-3-3972-8111; Fax: 81-3-3972-8666, Fukuda E-mail: fukuda.noboru@nihon-u.ac.jp, Abe E-mail: abe.masanori@nihon-u.ac.jp

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Abstract

Pyrrole-imidazole (PI) polyamides are novel gene silencers that strongly bind the promoter region of target genes in a sequence-specific manner to inhibit gene transcription. We developed a PI polyamide targeting human TGF-β1 (hTGF-β1). To develop PI polyamide targeting hTGF-β1 (Polyamide) as a practical medicine for progressive renal diseases, we examined the effects of Polyamide in two common marmoset models of nephropathy. We performed lead optimization of PI polyamides targeting hTGF-β1 by the dose-dependent inhibition of the PMA-stimulated expression of TGF-β1 mRNA in marmoset fibroblasts. Marmosets were housed with a 0.05% NaCl and magnesium diet and treated with cyclosporine A (CsA; 37.5 mg/kg/day, 8 weeks) to establish chronic nephropathy. Marmosets with nephropathy were treated with Polyamide (1 mg/kg/week, 4 weeks). We also established a unilateral urethral obstruction (UUO) model and examined the effects of Polyamide (1 mg/kg/week, 4 times) in marmosets. Histologically, the renal medulla from CsA-treated marmosets showed cast formation and interstitial fibrosis in the renal medulla. Immunohistochemistry showed strong staining of Polyamide in the renal medulla from CsA-treated marmosets. Polyamide treatment (1 mg/kg/week, 4 times) reduced hTGF-β1 staining and urinary protein excretion in CsA-treated marmosets. Polyamide reduced the glomerular injury score (GIS) and tubulointerstitial injury score (TIS) in UUO kidneys from marmosets. Polyamide significantly suppressed the hTGF-β1 and Snail mRNA expression in UUO kidneys from marmosets. PI polyamide effectively improved CsA- and UUO-associated nephropathy, indicating its potential application in the prevention of renal fibrosis in progressive renal diseases.

Keywords: human, TGF-β1, pyrrole-imidazole polyamide, renal disease, marmoset

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1. Introduction

The academic field "chemical genomics" is a chemistry-based field that involves research on genomics associated with genetic diagnostics, genome drug discovery, biochips, biomaterials, and other related topics. Thus, chemical genomics are expected to be the most important field for next-generation drug-discovery. Middle molecule compounds (molecular weight 500-4,000), including peptide compounds, have received attention as next-generation medicines. Middle molecule compounds have great potential as multipoint interaction with target genes and proteins in comparison to small molecule compounds. Pyrrole-imidazole (PI) polyamides, a type of middle molecule peptide, were discovered based on chemical genomics.

PI polyamides are DNA recognized peptide compounds that were initially identified from antibiotics such as distamycin A and duocarmycin A. PI polyamides consist of aromatic rings of amino acids N-methylpyrrole and N-methylimidazole that recognize and bind to DNA with sequence specificity [1,2]. Due to the ability of PI polyamides to form hydrogen bonds with high affinity and specificity to double stranded (ds)DNA, which is stronger in comparison to the bonds between protein and dsDNA. PI polyamides can inhibit protein interaction, including DNA transcription factors [3,4]. The recognition of dsDNA depends upon the pairing of Pyrrole (Py) and Imidazole (Im) side-by-side in the minor groove. The pairing of Im opposite Py will target the G-C base pair, whereas Py-Im pairing will target the C-G base pair. Py-Py pairing targets A-T and T-A base pairs and causes degeneration [2]. Structural models of the bonding of synthetic polyamide to dsDNA are shown in Figure 1. The synthetic polyamide was shown to bind to the appropriate B-form dsDNA in the minor groove [5]. Transcriptional regulation is essential for genetic expression. The initiation of transcription requires the binding of transcription factors to the cognate DNA response elements in the gene promoter. Figure 2 shows a principle for the suppression of gene transcription. PI polyamides bind to the minor groove and the block binding of transcription factors inhibits genetic expression. PI polyamides designed to bind transcription factor binding sites can potentially suppress genetic expression.

PI polyamides are fully resistant to the biological degradation induced by nucleases. PI polyamides do not require vector-assisted delivery systems because of their cell permeability, and they may easily enter into nuclei. Thus, the applicability of PI polyamides as novel gene therapy agents might be greater than that of nucleic acid

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medicines [6]. Synthetic PI polyamides that can target gene promoters might therefore be effective as practical medicines for regulating gene transcription.

TGF-β represents a large family of cytokines that are involved in the regulation of growth, differentiation, and morphogenesis in a wide range of cell types [7]. TGF-β1 is a multifunctional cytokine that is associated with cell growth, matrix formation, tissue repair, and inflammation [8]. We previously reported that PI polyamides targeting human TGF-β1 (hTGF-β1) promoter significantly inhibit both the TGF-β1 promoter activity and the expression of TGF-β1 mRNA and protein in human cells [9]. We have demonstrated that a PI polyamide targeting TGF-β1 effectively attenuates progressive renal diseases [10,11], stenosis of the carotid artery after angioplasty [12], alkali burn of the cornea [13], skin hypertrophic scar [14], liver fibrosis [15] and encapsulating peritoneal sclerosis [16] in rats.

Despite the availability of long-term therapies, progressive renal diseases caused by diabetic nephropathy, chronic glomerulonephritis and glomerulosclerosis cannot be cured through current treatments. Two hundred eighty thousand patients with end-stage renal failure undergo dialysis, and 37,000 patients newly receive dialysis every year in Japan. The medical expenses associated with dialysis represent a large problem for the medical economy. Thus, the development of a definitive treatment for chronic renal failure is a pressing issue. The PI polyamide targeting hTGF-β1 is a candidate compound as a definitive medicine for the treatment of progressive renal diseases.

In order to determine a lead compound among PI polyamides targeting human TGF-β1, we designed 7 PI polyamides to bind the hTGF-β1 promoter sequences [17]. We chose a lead compound based on the effects of these 7 PI polyamides on the hTGF-β1 mRNA expression in human vascular smooth muscle. We demonstrated that a relatively low dose of the lead compound completely inhibited the hypertrophic scar on the skin of common marmosets [18].

In the current preclinical study, we examined the effects of a PI polyamide targeting hTGF-β1 on the progression of renal injury in common marmosets in order to evaluate PI polyamides as practical medicines for the treatment of progressive renal diseases in humans.

2. Results

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2.1. Effects of PI polyamide targeting hTGF-β1 on the expression of TGF-β1 mRNA in marmoset fibroblasts in vitro

Concentrations of 10^{-9} and 10^{-7} M PI polyamide targeting hTGF-β1 (Polyamide) significantly ($p < 0.05$) inhibited the abundance of PMA-stimulated TGF-β1 mRNA on marmoset-derived fibroblasts. In contrast, mismatch PI polyamide (Mismatch) did not inhibit the abundance of PMA-stimulated TGF-β1 mRNA. These results indicate that Polyamide can inhibit the transcription of the TGF-β1 gene in marmoset fibroblasts (Figure 3).

2.2. Effects of Polyamide on fibrosis in CsA-induced nephropathy in marmosets

Figure 4 shows Masson trichrome staining of the renal medulla in marmosets injected with CsA with or without Polyamide. CsA (40 mg/kg) apparently enhanced Masson trichrome staining in the renal medulla in marmosets. Polyamide (1 mg/kg/week, for 5 weeks) inhibited staining in the renal medulla, indicating that Polyamide treatment effectively improved CsA-induced renal fibrosis.

2.3. Effects of Polyamide on the expression of TGF-β1 in CsA-induced nephropathy in marmosets

Figure 5 shows immunohistochemical staining of TGF- β1 in the renal medulla and cortex from marmosets injected with CsA and treated with or without Polyamide. CsA (40 mg/kg) apparently enhanced TGF-β1 staining in the renal tubules and glomeruli in marmosets. Polyamide (1 mg/kg/week, for 5 weeks) completely inhibited TGF-β1 staining in the renal medulla in marmosets.

2.4. Effects of Polyamide on renal degeneration in marmoset kidneys with unilateral urethral obstruction (UUO)

Figure 6 shows HE and Masson trichrome staining of the renal medulla in CUK and marmoset kidneys with UUO. Marmoset kidneys with UUO showed degeneration of the renal medulla consisting of interstitial fibrosis, tubular atrophy, and cast formation in contrast to the contralateral unobstructed kidney (CUK) (Fig. 6A). Polyamide (1 mg/kg/week, for 5 weeks) inhibited the degeneration and fibrosis of the kidney with UUO (Fig. 6d). Polyamide treatment did not significantly suppress GIS, whereas it

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significantly ($p < 0.05$) suppressed TIS in marmoset kidneys with UUO (Figs. 6B and 6C).

2.5. Effects of Polyamide on the expression of TGF-β1, α-smooth muscle actin (α-SMA) and E-cadherin in marmoset kidneys with UUO

Figure 7 shows immunohistochemical staining of TGF- β1, α-SMA and E-cadherin in the renal medulla from the CUK and UUO kidneys in marmosets. Immunostaining of TGF-β1 and α-SMA were enhanced in the renal medulla of the UUO kidney in comparison to the CUK kidney. Polyamide (1 mg/kg/week, for 5 weeks) completely inhibited the increased immunostaining of TGF-β1 and α-SMA in the renal medulla of the UUO kidney. In contrast immunostaining of E-cadherin was suppressed in the renal medulla of the UUO kidney in comparison to the CUK kidney. Polyamide (1 mg/kg/week, for 5 weeks) preserved the E-cadherin immunostaining in the renal medulla of the UUO kidney.

2.6. Effects of Polyamide on the expression of TGF-β1 and Snail mRNAs in marmoset kidneys with UUO

Figure 8 shows expression of TGF-β1 and Snail mRNAs in the renal medulla the from CUK and UUO kidneys in marmosets. The abundance of TGF-β1 ($p < 0.01$) and Snail ($p < 0.05$) mRNAs was significantly higher in the renal medulla of the UUO kidney in comparison to the renal medulla of the CUK kidney. After Polyamide treatment, the abundance of TGF-β1 and Snail mRNAs was significantly ($p < 0.01$) decreased in the UUO kidney in comparison to the untreated UUO kidney.

3. Discussion

In this study, we created two types of nephropathy in common marmosets: CsA-induced nephropathy and UUO nephropathy. It has been reported that CsA induces acute and chronic nephropathies. The acute nephropathy is reversible and induced by the contraction of afferent arterioles with an increase in intracellular calcium ion, increased endothelial damage due to the release of endothelin, and decreases in vasodilated prostaglandin E_2 and I_2 and nitric oxides [19]. The chronic administration of CsA mainly induces nephrotubular injury through increases in TGF-β1, and is associated with arteriole degeneration, focal glomerulosclerosis, and interstitial fibrosis due to

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hyperfiltration with a decreased number of nephrons [20,21]. These histopathological changes in CsA-induced nephropathy are known to be enhanced with lower blood levels of magnesium and sodium [22]. In this study, we therefore created a CsA-induced nephropathy in common marmosets fed a low sodium and magnesium diet. On the other hand, the spontaneous onset of nephropathy, such as the IgA nephropathy named Wasting marmoset syndrome, has been reported in individual common marmosets showing variable kidney degeneration [23]. In this study, Polyamide (1 mg/kg/week, for 5 weeks) effectively improved the CsA-induced renal fibrosis and completely inhibited TGF-β1 staining in the marmoset renal medulla.

We next investigated the effects of a PI polyamide targeting hTGF-β1 in marmoset kidneys with UUO, which is a standard model of renal fibrosis. In the UUO kidney, histological changes and immunostaining of target molecules can be compared to CUK kidney in the same animal. In the UUO kidney, renal tubules stimulates the production of TGF-β1, which induces the epithelial-mesenchymal transition (EMT) of the renal tubules and interstitial fibrosis [24,25]. In the present study, treatment with the PI polyamide targeting hTGF-β1 significantly suppressed the fibrosis of the renal medulla, with decreases in immunostaining of TGF-β1 and α-SMA, increased immunostaining of E-cadherin, and inhibited the expression of TGF-β1 and Snail mRNAs in marmoset kidneys with UUO. These findings indicate that PI polyamide targeting human TGF-β1 effectively suppress renal fibrosis and the EMT phenomenon in renal tubules in human nephropathies and that PI polyamide targeting human TGF-β1 has the potential to be developed as a practical medicine for human glomerulonephritis.

To evaluate the characteristics of PI polyamides as practical medicines, we investigated their pharmacokinetics, including the binding of PI polyamides to nuclei and chromosomes. FITC-labeled PI polyamide strongly localized to the nuclei in the renal tubules and glomeruli at 24 hours after injection in rats. The PI polyamide was also localized to the nuclei in the mid-layer smooth muscle of the aorta, lung and liver without any drug delivery system (DDS). An HPLC analysis of FITC-labeled PI polyamide also showed that PI polyamide was clearly detected in the urine, kidney and aorta, but not in the heart or brain [10]. Thus, PI polyamides themselves can be delivered to the organs and strongly bind to the nucleus, which is a potential advantage in comparison to nucleic acid medicines, such as siRNA and antisense DNA, which require certain DDSs. After the intravenous administration of FITC-labeled PI

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polyamides in rats, PI polyamides remained in the nuclei of the renal tubules on day 14, indicating that the “once a week” administration of PI polyamides may be effective for the treatment of progressive renal diseases [11]. In addition, we examined the binding of PI polyamide in chromosomal DNA. The binding pattern of the PI polyamide in the chromosomal DNA in HeLa cells was obviously different from the DAPI on the chromosome, indicating that the PI polyamides bind to the chromosome in a sequence-specific manner [11]. We then analyzed the pharmacokinetics of the PI polyamide. The pharmacokinetic parameters of PI polyamides are linear in the intravenous dose ranges, as revealed by the fact that the area under the blood concentration time curve (AUC) increased linearly as a function of the dose [26]. PI polyamides were mainly excreted in the urine, and low molecular weight PI polyamide was partially excreted in bile [27].

We performed a safety study of PI polyamides to investigate the lethal dose and side effects. The lethal doses of the polyamide were >40 mg/kg, and doses of <20 mg/kg were considered safe. Furthermore, PI polyamides did not show obvious side effects in mice. At 5 days after the injection of PI polyamide (20 mg/kg), there were no abnormalities in the serological data (e.g., blood cell counts, liver enzyme levels, total protein and lipid profiles). Next, we investigated the long-term effects of the 4-week administration of PI polyamide (1 mg/kg) on TGF-β1 in Dahl S-rats. Body weight and food consumption were not affected by the long-term administration of PI polyamide. Thus, these data indicate that the systemic administration of PI polyamides was not associated with any systemic side effects [11].

For the drug-discovery of DNA-recognized medicines, such as siRNA, antisense DNA and PI polyamides, off-target effects caused by binding to other genes and suppressing the function of non-target genes represent a large problem. To confirm the specificity of PI polyamides targeting the TGF-β1 promoter, we previously performed a microarray analysis of the kidney in Dahl-S rats treated with PI polyamide targeting TGF-β1 [11]. In the analysis, the PI polyamide affected only 3% of the 430,000 interrogated kidney transcripts from Dahl-S rats. We performed a further analysis of the promoter structure in these inhibited genes for the presence of AP-1 sites and PI polyamide-binding sites. There were only a few common sites of AP-1 and PI polyamide binding in the 3 kb upstream of the transcription initiation sites in these inhibited genes, which were all TGF-β1-related molecules. We therefore assumed that

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the PI polyamide particularly suppressed the TGF-β1 gene at the transcription level because we designed a PI polyamide spanning the transcription binding sequences and target gene-specific sequences. These findings indicate the high specificity of PI polyamide to the target gene.

Recently some groups have developed alkylating PI polyamides as anticancer agents [28-30]. DNA alkylating agents can damage cellular DNA and lead to anticancer activity. Since the non-specific DNA alkylation damages normal cells, alkylating PI polyamides can damage cancer cells in sequence-specific alkylation for cancer genes [31]. However, the off-target effects of the alkylating PI polyamides by binding and alkylating to non-target genes have been concerned as practical medicines. On the other hand, we have developed non-alkylating PI polyamides as a gene silencer to block transcription factor binding on the target gene. Transcriptional silencing PI polyamides may also have off-target effects by non-specific binding on a non-target gene. However, transcriptional silencing PI polyamides simply suppress the transcriptionally activated gene function in a disease state, even when binding to non-target genes, indicating that the non-alkylating PI polyamides might have low side effects and high specificity.

In conclusion, in the present study we examined the effects of low-dose treatment with a PI polyamide targeting hTGF-β1 that was injected once a day in common marmoset models of chronic nephropathy induced by CsA and UUO, as a preclinical study. The synthetic PI polyamide targeting hTGF-β1 effectively improved nephropathy in common marmosets. These results indicate that the PI polyamide will be developed as a medicine that may provide a radical cure for progressive renal diseases.

4. Materials and Methods

4.1. Design and synthesis of PI polyamides to hTGF-β1 promoter

Figure 2A show the results of an NCBI BLAST Two Sequence Analysis of the hTGF-β1 promoter sequence, which demonstrated 86% sequence homology between humans and common marmosets. We designed a PI polyamide (Polyamide) to bind bp 819-826 adjacent to the adipocyte P2 gene, which contains a regulatory element (FSE2) binding site of the hTGF-β1 promoter sequence (Figure 2B). The binding sequence of Polyamide was completely identical to the human and marmoset TGF-β1 promoter sequences. The structures of Polyamide and mismatch PI polyamides are shown in

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Figure 2C. Mismatch PI polyamide (Mismatch) had an identical structure and molecular weight to Polyamide.

We induced Im and Py substitution to create the PI polyamides. Machine-assisted automatic synthesis of hairpin-type PI polyamides was carried out with the use of a continuous-flow peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan) at 0.1 μmol scale (200 mg of Fmoc-b-alanine-CLEAR Acid Resin, 0.50 meq/g, Peptide Institute, Osaka, Japan). Automatic solid phase synthesis was performed by washing with dimethylformamide (DMF), removing the Fmoc group using 20% piperidine/DMF, washing with methanol, coupling with a monomer for 60 min in an environment of 1-[bis(dimethylamino)methylene]-5-chloro-1H-benzotriazolium 3-oxide hexafluorophosphate (HCTU) and diisopropylethylamine (4 eq each), washing with methanol, protecting with acetic anhydride/pyridine, and washing with DMF as the final step. After the Fmoc group was removed from the Fmoc- β-alanine-Wang resin, successive washes of the resin were performed with methanol. The coupling step was carried out with Fmoc-amino acid, followed by a methanol wash. We repeated these steps until the entire sequencing was complete. After completion of the coupling steps, the N-terminal amino group was protected and washed with DMF, then the reaction vessel was drained. We next isolated the synthetic polyamides after the cleavage step (5 ml of 91% trifluoroacetic acid-3% triisopropylsilane-3% 5 dimethylsulfide-3% water/0.1 mmol resin) by cold ethyl ether precipitation. The synthetic polyamides were isolated after the cleavage step (5 ml of N, N-dimethylaminopropylamine/0.1 mmol resin, 50°C overnight) by cold ethyl ether precipitation. To purify the polyamides, high-performance liquid chromatography (HPLC) was performed using a PU-980 HPLC pump, a UV-975 HPLC UV/VIS detector (Jasco, Easton, MD), and a Chemcobound 5-ODS-H column (Chemco Scientific, Osaka, Japan).

4.2. Cell culture

Marmoset fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) and 50 mg/ml streptomycin (Invitrogen). After it reached confluence (7-10 days after seeding 1 vial of 10^5 cells/cm^2), the VSMC culture displayed a typical hill-and-valley pattern. Marmoset fibroblasts were prepared from newborn skin by digestion with 5 mg/ml collagenase type I (Sigma, St. Louis, MO) overnight. They were cultured in DMEM

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supplemented with 10% fetal calf serum, 0.1 mg/ml penicillin and 0.05 mg/ml gentamicin. Cells were passaged by trypsinization with 0.05% trypsin (Gibco Life Technologies, Gaithersburg, MD) and plated in 6- or 24-well culture dishes at a density of 10^5 cells/cm^2. Cells were cultured in a water-saturated CO_2 incubator at 37°C.

4.3. RNA extraction and real-time PCR

TRIzol reagent (Invitrogen, CA) was used to extract total RNA from cultured cells. The total RNA (1 μg) was then reverse transcribed into cDNA with random 9-mers with a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio, Ohtsu, Japan). Assay-on-Demand primers and probes (human TGF-β1: Hs00998133_m1) were obtained from Applied Biosystems Life Technologies (Tokyo, Japan). An ABI Prism 7300 (Applied Biosystems) was used to quantify mRNA. Each sample (each reaction, 5 μl complementary DNA; total volume, 25 μl) was run in triplicate. To control sample loading, we determined 18S ribosomal RNA levels with TaqMan Ribosomal RNA Control Reagents (Applied Biosystems). The amplification conditions were as follows: 50°C for 2 min, 95°C for 10 min, 60 cycles of denaturation (95°C for 15 sec) and combined annealing-extension (60°C for 1 min). We determined the threshold cycle (Ct) and calculated the relative quantification of the marker gene mRNA expression using the comparative Ct method.

4.4. Ethics

This study conformed to the standards of the US National Institute of Health's *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). The Nihon University IACUC committee approved this study (approval code: AP13D009), which was conducted in accordance with the Guidelines for Conducting Animal Experiments of the CIEA.

Fifteen male common marmosets (Callithrix jacchus) obtained from CLEA Japan, Inc. (Tokyo, Japan) were used in this study. Experiments were performed in the Central Institute for Experimental Animals (CIEA). The marmosets were housed in pairs in stainless steel living cages (39655670 cm) with 45–55% humidity and illumination for 12 hours per day. Wood perches for locomotion and a platform for a bed were placed in each cage for environmental enrichment. Marmosets were kept healthy and nourished with a balanced diet (CMS-1M; CLEA Japan Inc.), including

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mixed L(+)-ascorbic acid (Nacalai Tesque, Tokyo, Japan), vitamins A, D3, and E (Duphasol AE3D; Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan), and honey (Nihonhatimitsu Co., Ltd., Gifu, Japan). The marmosets were fed with chow moistened with hot water in the morning and dry chow in the afternoon. In addition to the normal diet, the marmosets were fed with sponge cakes, biscuits or apple jelly when in contact with humans. Each of the cages contained puzzle feeders. The had *ad libitum* access to tap water from feed valves. No marmosets were sacrificed for the experiments involving skin hypertrophic scar creation.

4.5. Creation of nephropathy in marmosets and treatment with PI polyamides

We created two types of nephropathy in marmosets. Male marmosets weighing 300 to 350 g were housed with a 0.05% NaCl and 0.05% magnesium diet and were subcutaneously injected with 40 mg/kg/day cyclosporine A (CsA, Novartis Pharmaceuticals, Basel, Switzerland) for 8 weeks to establish chronic nephropathy. Marmosets with CsA-nephropathy were intravenously injected with Polyamide (1 mg/kg/week, for 4 weeks) from 4 weeks after the start of CsA injection. To create the UUO model, marmosets were pre-anesthetized with an intramuscular injection of medetomidine (0.04 mg/kg; Nippon Zenyaku Kogyo, Koriyama, Japan), midazolam (0.40 mg/kg; Astellas Pharma, Tokyo, Japan), and butorphanol (0.40 mg/kg; Meiji Seika Pharma, Tokyo, Japan). Ampicillin (15 mg/kg; Meiji Seika Pharma) was also administered and the animals were hydrated subcutaneously with 2 ml/head of fluid (KN No.1 injection; Otsuka Pharmaceutical, Tokyo, Japan). The animals were then anesthetized by inhalation of 1.0–3.0% isoflurane (Abbott Japan, Tokyo, Japan) via a ventilation mask. In marmosets undergoing UUO, the left ureter was ligated with 6-0 silk at two points and then severed between the ligatures. In the sham-operated marmosets, the left ureter was left undisturbed. The marmosets with UUO were intravenously injected with Polyamide (1 mg/kg/week) through the tail vein for 4 weeks from 1 week after the operation. Control marmosets with UUO were intravenously injected with same amount of saline through the tail vein.

4.6. Histopathological and immunohistochemical examinations

Tissue samples from the kidney were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Sections of 4 μm in thickness were then stained with

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hematoxylin-eosin or Masson trichrome. To quantify the amount of matrix in the glomeruli, 50 glomeruli in each section were randomly selected. The percentage of each glomerulus occupied by mesangial matrix was estimated and assigned a score of 0 to 4 as follows: 0, normal; 1, involvement of up to 25% of the glomerulus; 2, involvement of 25–50% of the glomerulus; 3, involvement of 50–75% of the glomerulus; or 4, involvement of 75–100% of the glomerulus. Grading for the glomerular injury score (GIS) was performed as described previously [32]. To quantify the tubulointerstitial area, 20 areas in each renal cortex were randomly selected. The tubulointerstitial injury score (TIS) graded basement membrane thickening, dilation, atrophy, interstitial inflammation, interstitial fibrosis, tubular necrosis, desquamation, and hydropic degeneration as follows: grade 0, none; grade 1, <10%; grade 2, 10–25% as above; grade 3, 26–50%; grade 4, 51–75%; and grade 5, >75% in an average of 20 fields per kidney coronal section.

Sections (thickness: 4 μm) were deparaffinized, dehydrated in a routine manner and incubated overnight at 4°C with TGF-β1 anti-human TGF-β1 rabbit polyclonal antibody (Yanaihara, Shizuoka, Japan) (1:1000). For TGF-β1 staining, sections were incubated with horseradish peroxidase-conjugated anti-biotin labeling solution (ABC Elite Kit, Vector) for 30 min at 22°C, followed by washing and incubation with 3,3'-diaminobenzidine (DAB) solution. The sections (thickness: 4 μm) were incubated overnight at 4°C with anti-human E-cadherin rabbit polyclonal antibody (Abcam, Tokyo, Japan) (1:5) or anti-human α-SMA monoclonal mouse antibody (DAKO, Tokyo, Japan) (1:1000). For E-cadherin and α-SMA staining, sections were incubated with peroxidase labeled mouse and rabbit polyclonal goat antibody (Sigma-Aldorich, Tokyo, Japan). Counterstaining was then performed before examination under a light microscope.

4.7. Statistical Analysis

Values are shown as the mean ± SE. We used Student's *t*-test for to analyze unpaired data and a two-way ANOVA with the Bonferroni/Dunn procedure as a post hoc test. P values of < 0.05 were considered to indicate statistical significance.

5. Conclusions

The synthetic PI polyamide targeting hTGF-β1 effectively improved nephropathy induced by CsA and UUO in common marmosets as preclinical study. These results indicate that the PI polyamide can be developed as a practical medicine for progressive renal diseases.

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Disclosures

None.

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Figure Legends

Figure 1.

(A) Putative binding structures of double stranded DNA with pyrole-imidazole (PI) polyamide targeting human transforming growth factor-β1. DNA complexes were calculated to minimize by InsightII/Discover. DNA is indicated by grey color. PI polyamides are indicated by orange color. From Ref [5]. (B) Principle of transcriptional suppression of genes. PI polyamides bind to the target promoter region of targeting genes by hydrogen binding to prevent binding of the transcription factors to transcriptionally suppress the gene expression.

Figure 2.

(A) The sequences of human and marmoset transforming growth factor (TGF)-β1 promoter analyzed by an NCBI BLAST Two-Sequence Analysis. Binding sites (box) of pyrrole-imidazole (PI) polyamide on the human and marmoset TGF-β1 promoter. (B, C) The structures and target sequences of PI polyamides targeting the human TGF-β1 promoter and the structure of the mismatch polyamide. Open circle indicates pyrrole. Closed circle indicates imidazole.

Figure 3.

The effects of pyrrole-imidazole (PI) polyamides targeting the human transforming growth factor (TGF)-β1 promoter (Polyamide) and mismatch polyamide (Mismatch) on the expression of TGF-β1 mRNA in marmoset fibroblasts. Marmoset fibroblasts were incubated with 10^{-9} and 10^{-7} M Polyamide and Mismatch in the presence or absence of 10^{-6} M phorbol 12-myristate 13-acetate (PMA). Total RNA was extracted, and the expression of TGF-β1mRNAs was evaluated by real-time polymerase chain reaction. Data indicate the mean ± SEM (n = 4). * $p < 0.05$ vs. PMA without PI polyamide. # $p < 0.05$ vs. without PMA.

Figure 4.

The effects of pyrrole-imidazole polyamide targeting human TGF-β1 (Polyamide) on fibrosis in marmosets with cyclosporin A (CsA)-induced nephropathy. Male marmosets

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were housed with a 0.05% NaCl and 0.05% magnesium diet and subcutaneously injected with CsA (40 mg/kg/day) for 8 weeks. Marmosets with CsA-nephropathy were intravenously injected with Polyamide (1 mg/kg/week, for 4 weeks) from 4 weeks after the start of CsA injection. Sections (thickness: 4 μm) of kidney tissue samples were stained with hematoxylin-eosin (HE) and Masson trichrome (Masson).

Figure 5.

The effects of pyrrole-imidazole polyamide targeting human TGF-β1 (Polyamide) on the expression of TGF-β1 in CsA-induced nephropathy in marmosets. Male marmosets were housed with a 0.05% NaCl and 0.05% magnesium diet and subcutaneously injected with CsA (40 mg/kg/day) for 8 weeks. Marmosets with CsA-nephropathy were intravenously injected with Polyamide (1 mg/kg/week) for 4 weeks from 4 weeks after the start of CsA injection. Sections (thickness: 4 μm) were incubated with TGF-β1 anti-human TGF-β1 antibody and then with horseradish peroxidase-conjugated anti-biotin labeling solution.

Figure 6.

The effects of pyrrole-imidazole polyamide targeting human TGF-β1 (Polyamide) on renal degeneration in unilateral urethral obstruction (UUO) kidney in marmosets. The left ureter was ligated, whereas in sham-operated marmosets, the left ureter was left undisturbed. The marmosets with UUO were intravenously injected with Polyamide (1 mg/kg/week) through the tail vein for 4 weeks from 1 week after the operation. Control marmosets with UUO were intravenously injected with same amount of saline through the tail vein. (A) Paraffin-embedded sections of the removed renal cortex were stained with hematoxylin and eosin (HE) and Masson trichrome (Masson) in the contralateral unobstructed kidney (CUK) and UUO kidney. (B) Glomerular injury score (GIS). (C) Tubulointerstitial injury score (TIS). Data indicate the mean ± SEM (n = 6). * p <0.05 in the indicated columns. Bar = 50 μm.

Figure 7.

The effects of P pyrrole-imidazole polyamide targeting human TGF-β1 (Polyamide) on the expression of TGF-β1, α-SMA and E-cadherin in the kidney with unilateral urethral obstruction (UUO) in marmosets with UUO. The left ureter was ligated, whereas in the

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sham-operated marmosets, the left ureter was left undisturbed. Marmosets with UUO were intravenously injected with Polyamide (1 mg/kg/week) through the tail vein for 4 weeks from 1 week after the operation. CUK, contralateral unobstructed kidney. Control marmosets with UUO were intravenously injected with the same amount of saline through the tail vein.

Figure 8.

The effects of P pyrrole-imidazole polyamide targeting human TGF-β1 (Polyamide) on the expression of TGF-β1 and Snail mRNAs in the kidney with unilateral urethral obstruction (UUO) in marmosets with UUO. The left ureter was ligated, whereas in the sham-operated marmosets, the left ureter was left undisturbed. Marmosets with UUO were intravenously injected with Polyamide (1 mg/kg/week, for 4 weeks) through the tail vein from 1 week after the operation. Control marmosets with UUO were intravenously injected with same amount of saline through the tail vein. CUK, contralateral unobstructed kidneys. Data indicate the mean ± SEM (n = 6). *p <0.05 and ** p <0.01 in the indicated columns.

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Figure 1.

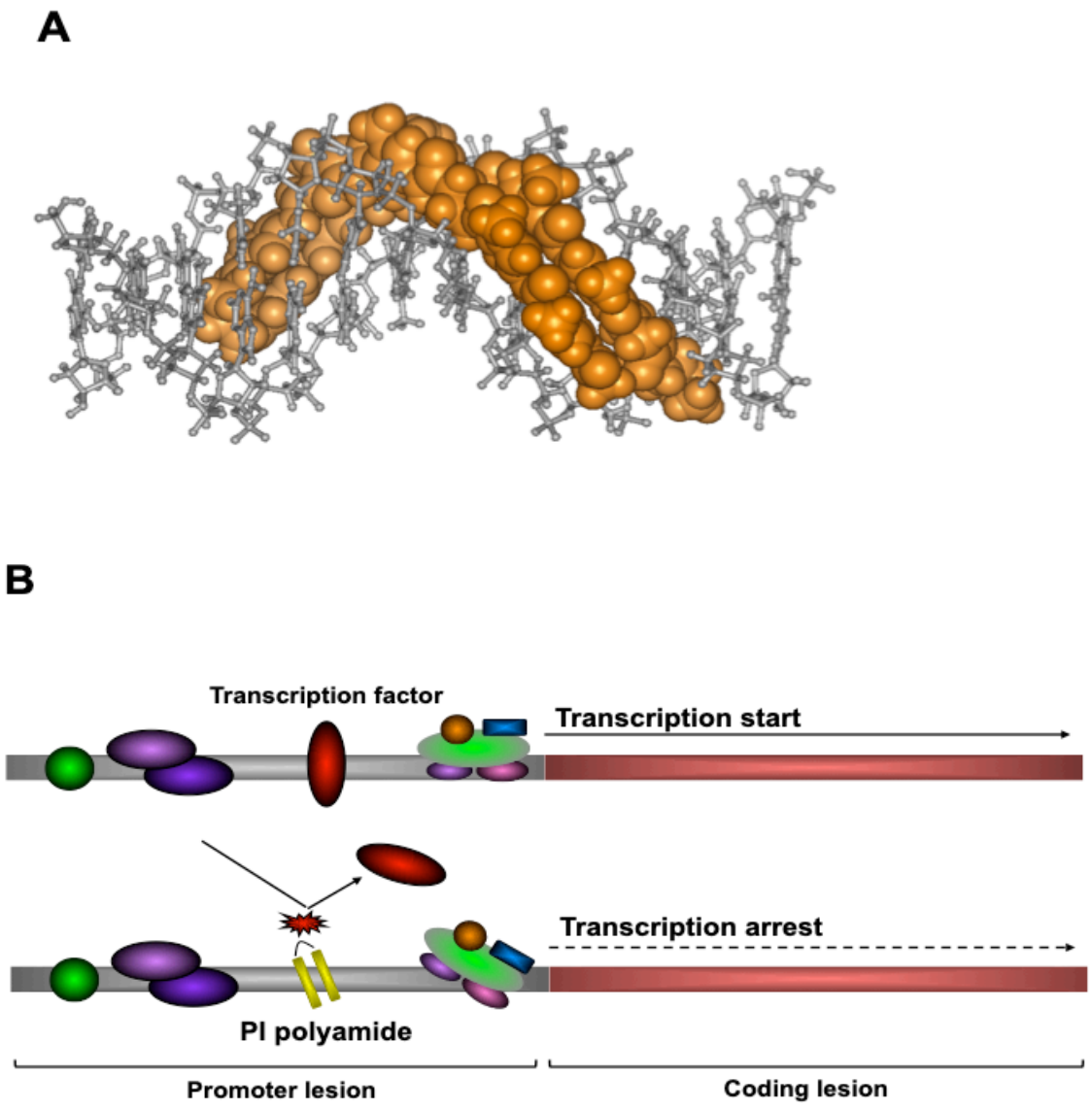

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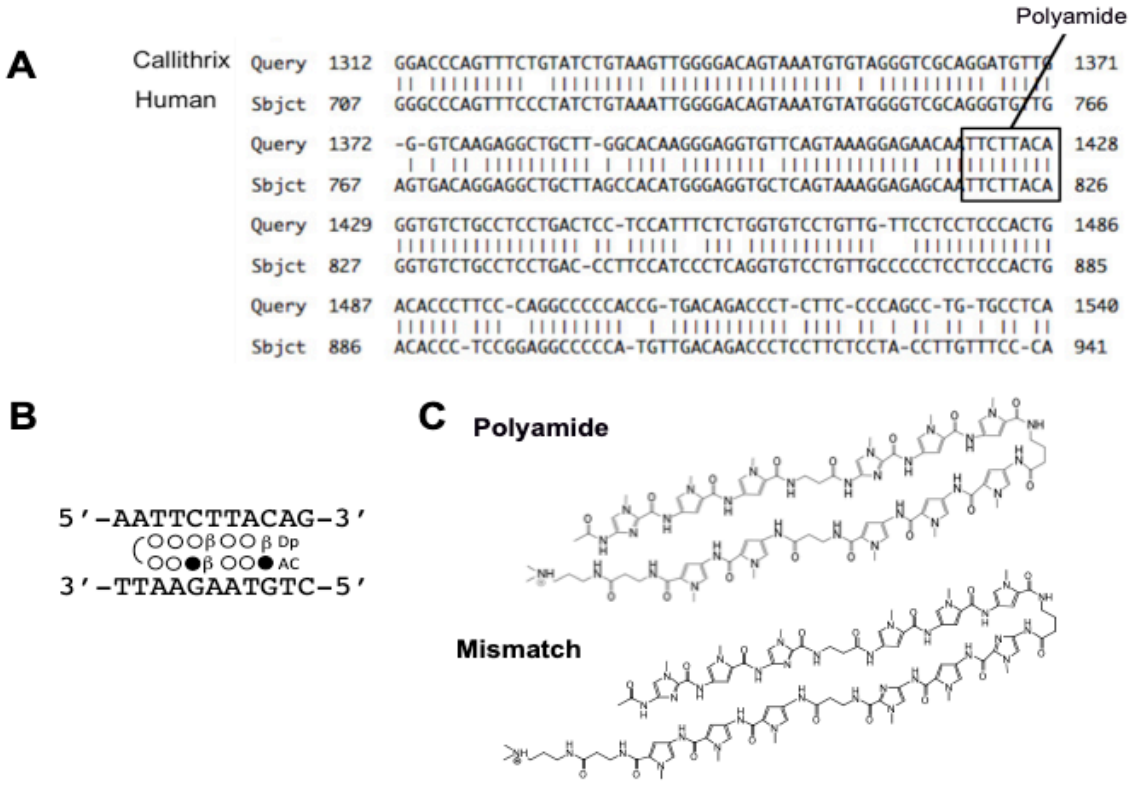
Figure 2.



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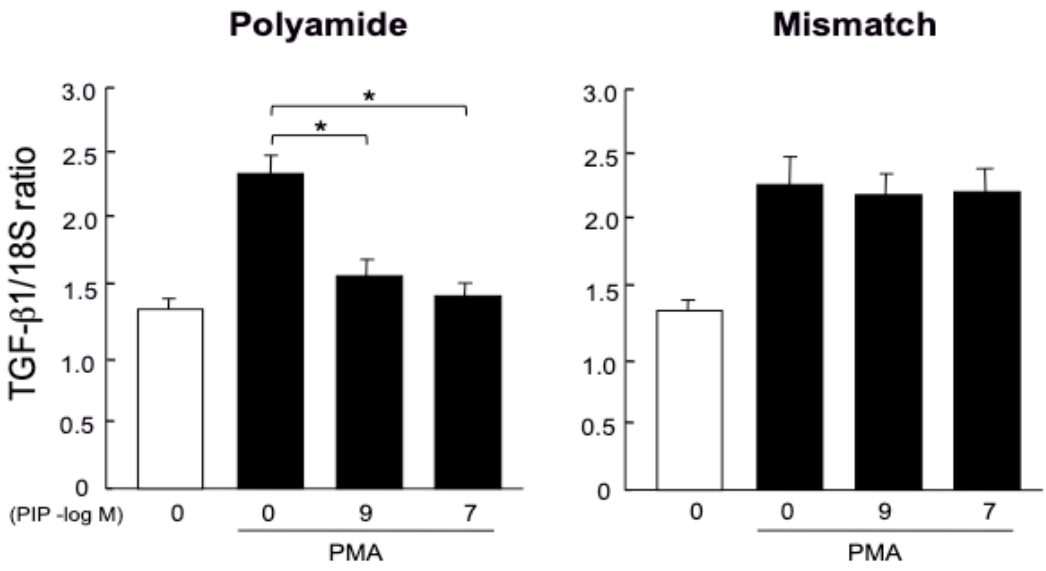

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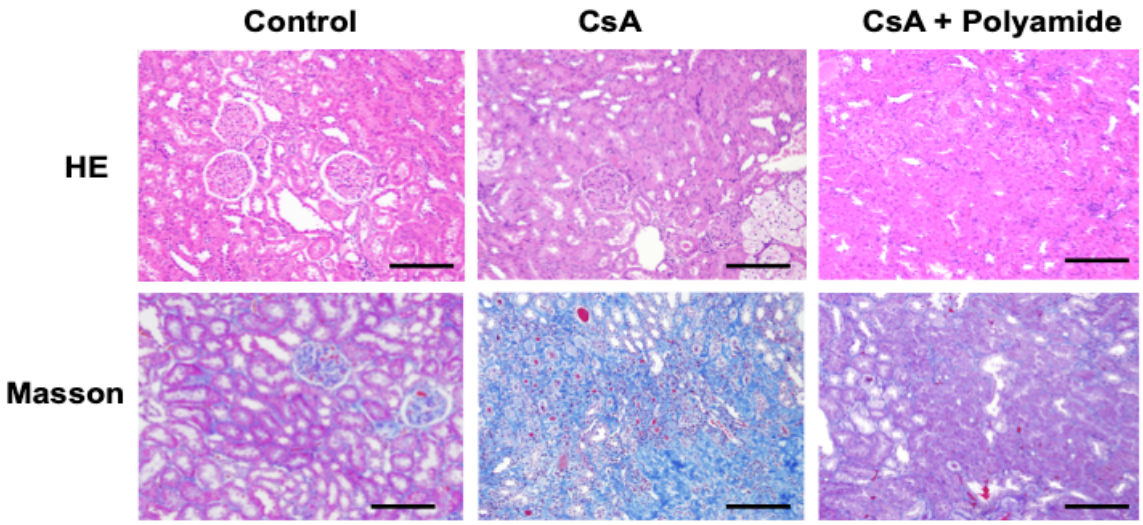
Figure 4.



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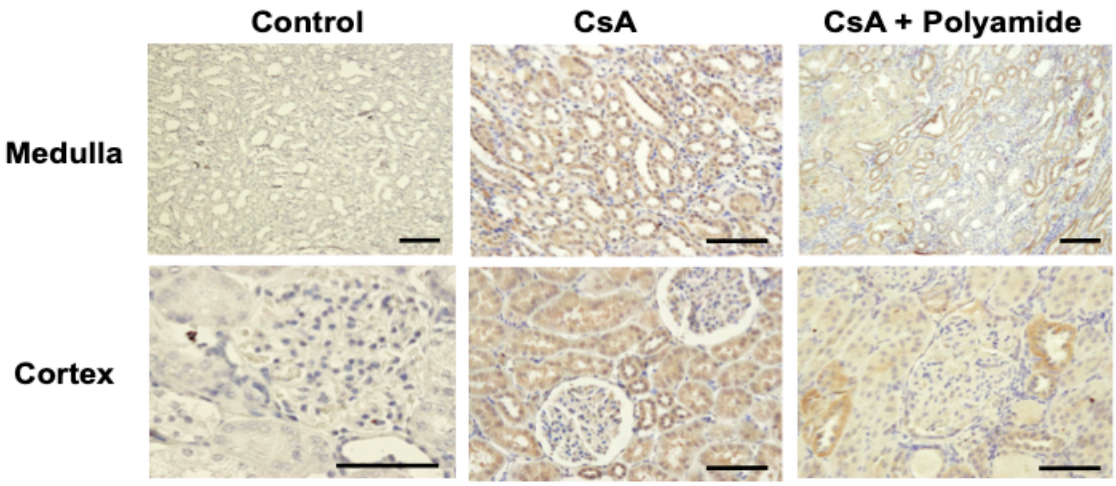

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Figure 6.

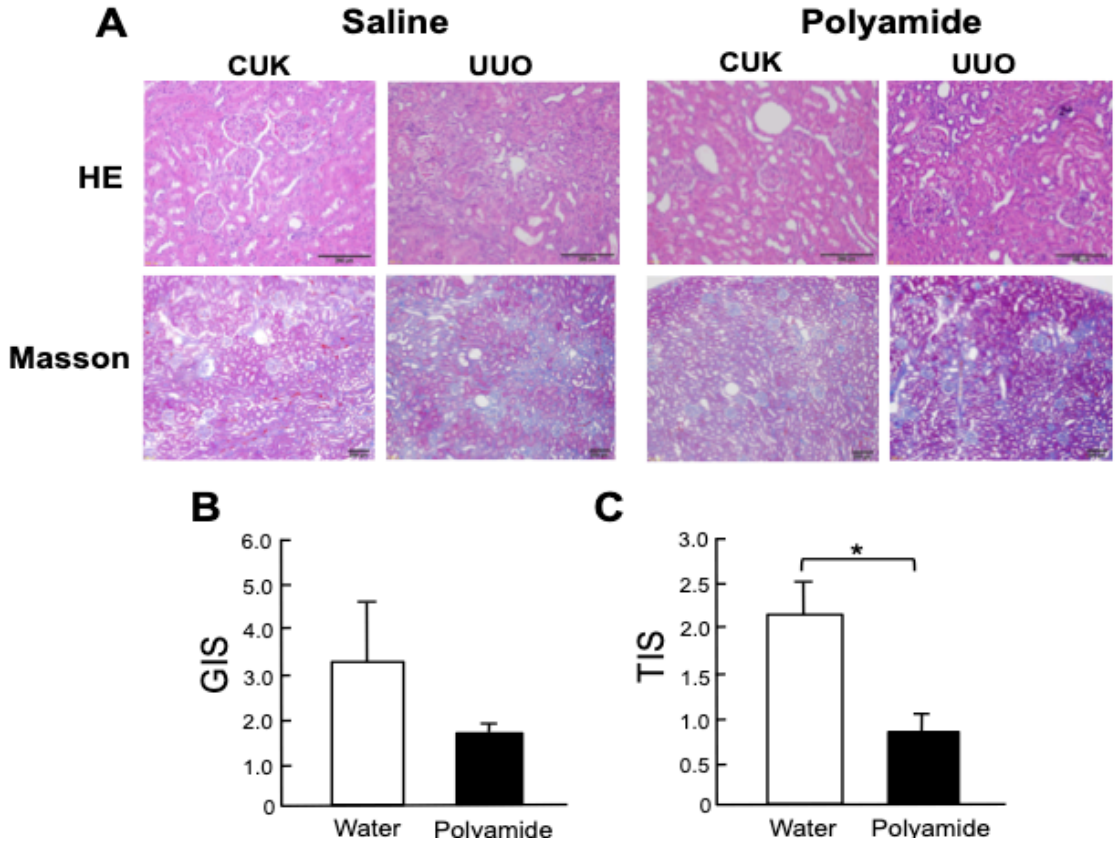

Figure 7.

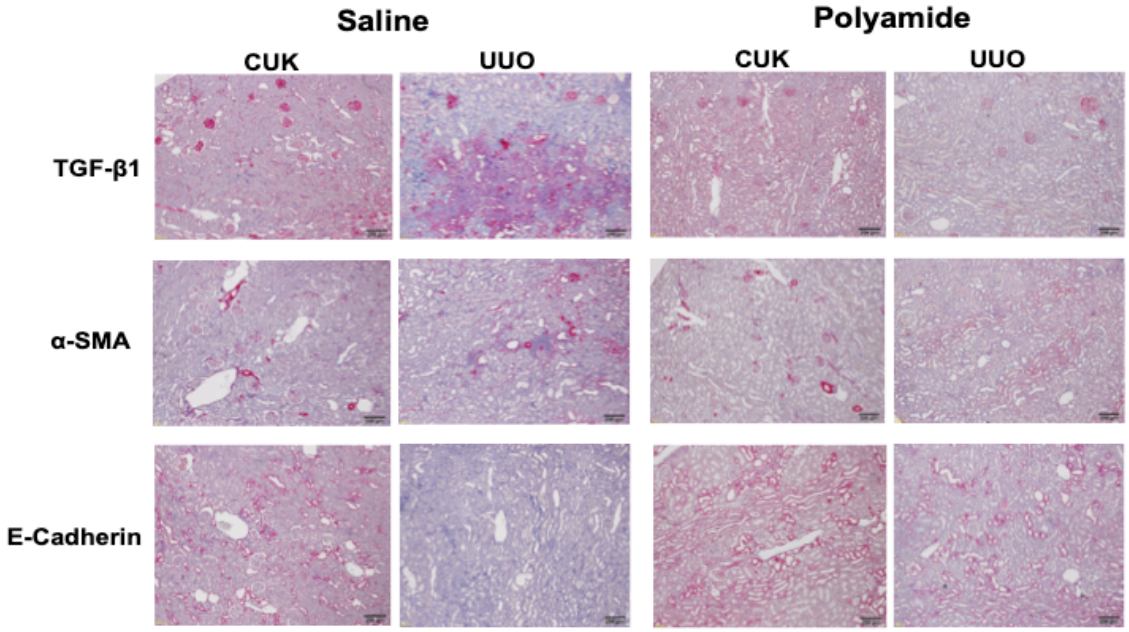

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Figure 8.