Development of Pyrrole-Imidazole (PI) Polyamides as a Novel Gene Silencer by Transcriptional Regulation

Gentier Biosystems Inc./Central Research Co.,Ltd

Summary

PI polyamides are completely novel medicines that strongly bind dsDNA in a sequence specific manner to silence gene expression by blockade of the transcription factors. PI polyamides are resistant to nucleases and delivered into any tissues without DDS. We have developed PI polyamides targeting human TGF-β1, CTGF and LOX-1 at the preclinical stages using marmoset. Lethal doses of PI polyamide are over 40 mg/kg. PI polyamides are excreted mainly into urine. PI polyamides targeting human TGF-β1 will be systematically administered i.v. or i.m. once in a week for progressive renal diseases, pulumonary fibrosis and liver chirrohosis. We have developed an automatic technology to synthesize PI polyamides and tried to large scale synthesis PI polyamides in GMP grade.

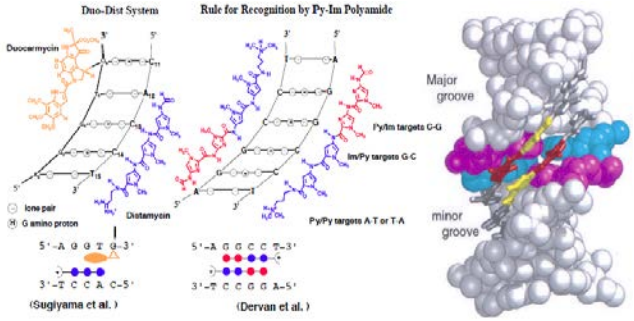
Proposal

We would propose followings:

1) Research collaboration in the drug discovery of PI polyamides targeting human TGF-β1, CTGF and LOX-1 with Nihon University.
2) Financial support for the drug discovery and the large scale synthesis in GLP and GMP grade.
3) Investment for Gentier Biosystems Inc. a venture company having patents for the synthesis technology and human TGF-β1, CTGF and LOX-1 PI polyamides.

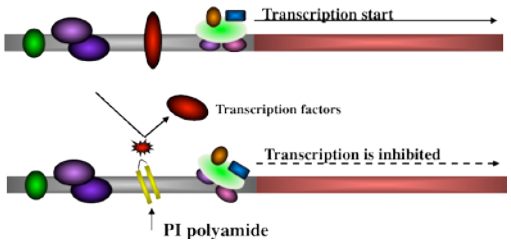
Principle of the gene silencing by PI polyamides

PI polyamides are small synthetic molecules composed with the aromatic rings of the N-methylpyrrole and N-methylimidazole amino acid (Trauger et al. 1996, Sugiyama et al. 1996).



Synthetic polyamides can bind to specific nucleotide sequences in the minor groove of double-helical DNA with high affinity and specificity, suggesting that PI polyamides could be useful tools for molecular biology and novel medicines. Binding site specificity is dependent on the side-by-side pairing of Pyrrole (Py) and imidazole (Im): the Py/Im pair targets the CG base pair, Im/Py recognizes the GC base pair, and Py/Py binds both AT and TA base pairs. The hairpin motif. The amino and carboxy terminus of the antiparallel dimers are connected by γ-aminobutyric acid (Dervan. 2001).

Transcriptional regulation is essential for gene expression. Initiation of transcription requires binding of transcription factors to the cognate DNA response elements in the gene promoter. PI polyamides bind the minor groove and block binding of transcription factors inhibiting gene expression. PI polyamides designed to bind transcription factor binding sites can potentially suppress gene expression.



Properties of PI polyamides as the gene silencing medicines

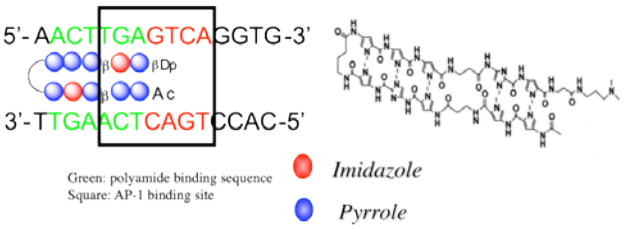
1. PI polyamides are complete novel medicines as the gene silencer.
2. PI polyamides are small synthetic molecules and resistant to nucleases compared to nucleic acid medicine such as antisense DNA, ribozyme, and siRNA.
3. PI polyamides recognize and bands target dsDNA in a sequence specific manner.
4. PI polyamides can be delivered into tissues and cells without any vectors.
5. PI polyamides inhibit gene expression by blockade of the transcription factors binding to scilence gene, not to knockdown gene, suggesting low adverse effects.
6. PI polyamides can be freely designed to any target genes which are repsponsible in the intractable diseases.
7. PI polyamides will be peroral gene therapy agents.

TGF-β1 is involved in several diseases

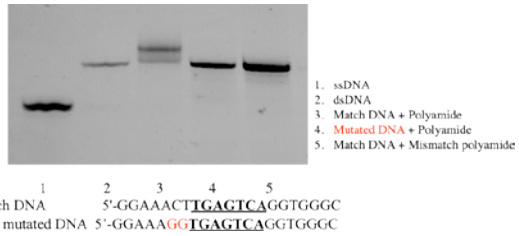
TGF-β1 represents a large family of cytokines that are involved in the regulation of growth, differentiation, and morphogenesis in a wide range of cell types (Sporn MB and Roberts AB. 1992). TGF-β is a multifunctional protein that regulates cell growth, differentiation, motility, and extracellular matrix production in the normal wound healing process, but has also been

implicated in excessive scar formation and fibrotic disorders (Leask A. et al. 2004). TGF-β1 plays a pivotal role in chronic inflammatory changes of the interstitium and accumulation of extracellular matrix during renal fibrogenesis, coronary stenosis, hypertropic scar of skin, eye cornea injury, peritoneum sclerosis, liver cirrhosis and lung fibrosis.

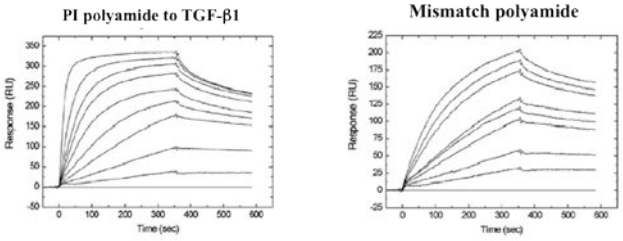
Designing a PI polyamide targeting TGF-β1 and its binding to dsDNA



To design a polyamide targeting TGF-β1, we analyzed the TGF-β1 promoter structure and PMA-stimulated activity in promoter deletion mutants. Positive-regulatory elements stimulated by PMA were found at upstream which also contain an AP-1 binding site. AP-1 elements respond to AP-1 transcription factors such as Jun homodimers or Fos/Jun heterodimers, PMA, angiotensin II, and v-Src. Stimulation of TGF-β1 promoter activity occurs by binding to the AP-1 element in hypertension. Polyamide targeted to TGF-β1 dared to be designed not to cover AP-1 consensus sequences but spans the boundary of AP-1 binding site with the intention of obtaining specificity to the promoter (Matsuda et al. 2006).



This polyamide showed strong and specific binding to the target DNA in gel mobility shift. This polyamide bound the appropriate 21-bp double-stranded DNA but did not bind the 2-bp mutated DNA, whereas Mismatch did not show binding to appropriate DNA.

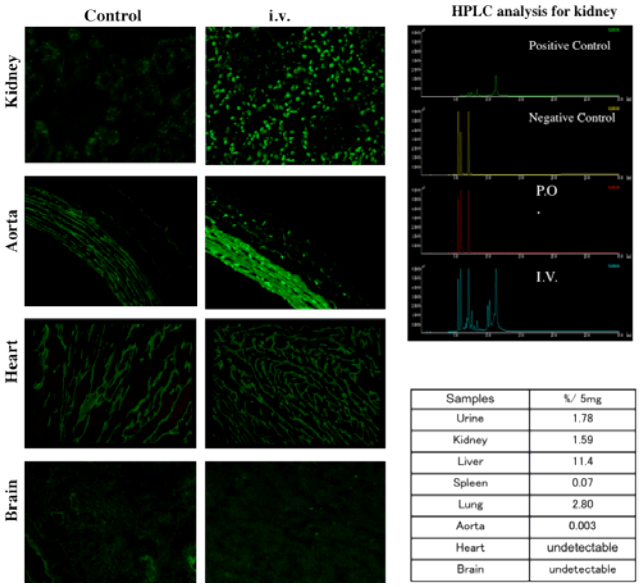


	KD (M)	k_a (1/Ms)	k_d (1/s)	Specificity
Polyamide binding	1.43×10^{-9}	5.97×10^{5}	8.54×10^{-4}	685
Mismatch binding	9.76×10^{-7}	7.68×10^{2}	7.49×10^{-4}	

Kinetics of polyamide and mismatch bindings with target double-strand DNA obtained from Biacore assay. Fast binding of polyamide to the target sequence occurred relative to that of mismatch to allow match binding to reach equilibrium at highconcentrations. Specificity of Polyamide to targeting dsDNA is 685 times to Mismatch polyamide (Matsuda et al. 2006).

PI polyamide can be delivered into several organs by systematic administration without vectors

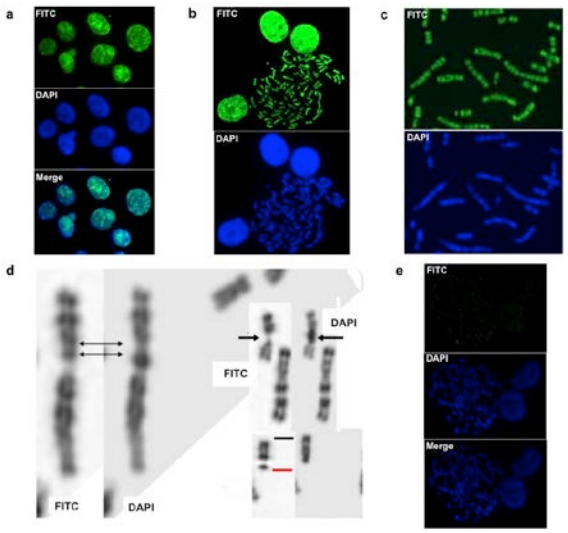
Five mg FITC-labeled PI polyamide was injected into Wistar rats via the tail vein. After 24 h, organs removed each tissue was homogenized, centrifuged and freeze-dried. Urine was also collected for 24 h in metabolic cages and all samples were subjected to HPLC. The *in vivo* distribution of PI polyamide in the kidney, aorta, heart, and brain 24 h after intravenous injection is shown. FITC-labeled PI polyamide strongly localized to nuclei in nephrotubullus and glomeruli. PI polyamide was also localized to nuclei in midlayer smooth muscle of the aorta, lung and liver. PI polyamide was not considerably distributed in heart or brain. HPLC analysis of FITC-labeled PI polyamide also shows that PI polyamide was clearly detected in urine, kidney and aorta but not in heart or brain (Matsuda et al. 2006).



Samples	%/ 5mg
Urine	1.78
Kidney	1.59
Liver	11.4
Spleen	0.07
Lung	2.80
Aorta	0.003
Heart	undetectable
Brain	undetectable

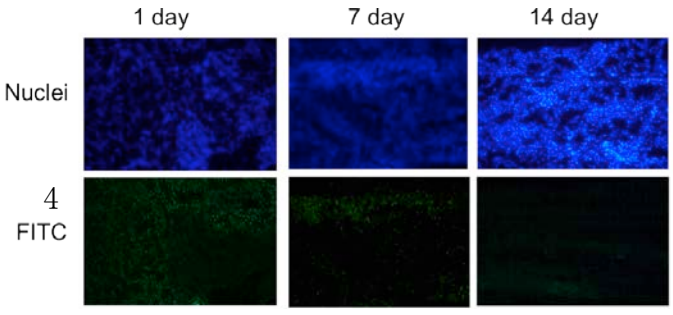
Binding of PI polyamide in chromosomal DNA

Binding pattern of PI polyamide in chromosomal DNA in HeLa cells was obviously differed from DAPI on chromosome, indicating PI polyamides bind to the chromosome in the sequence specific manner (Matsuda et al. 2011).



PI polyamides bind tissue neucleus for one week

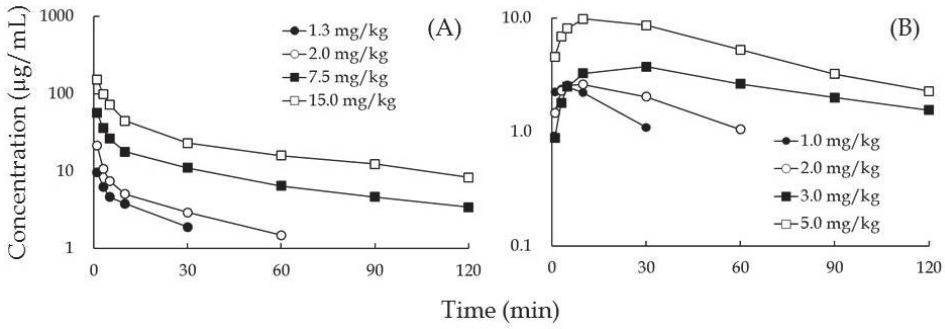
After intra venous administration of FITC labeled PI polyamide, PI



polyamides still remain in nuclei of renal tubules at day 14, indication that administration of PI polyamides as medicine is estimated the “once a week” (Matsuda et al. 2011).

Pharmacokinetic analysis of PI polyamides

The mean plasma concentration-time profiles after the intravenous administration of PI polyamide A (MW 1,000) at 1.3, 2.0, 7.5, and 15.0 mg/kg and after that of PI polyamide B (MW 1,700) at 1.0, 2.0, 3.0, and 5.0 mg/kg are shown in below figures (A) and (B) (Fukusawa et al. 2009).



The pharmacokinetic parameters of PI polyamides A and B obtained in rats using noncompartmental analysis are summarized in Tables (below). The pharmacokinetics of PI polyamides A and B are linear in the intravenous dose ranges of 1.3-15.0 mg/kg and 1.0-5.0 mg/kg, respectively as revealed by the fact that AUC increased linearly as a function of dose, and CLt and Vss remained unaltered (Nagashima et al. 2009).

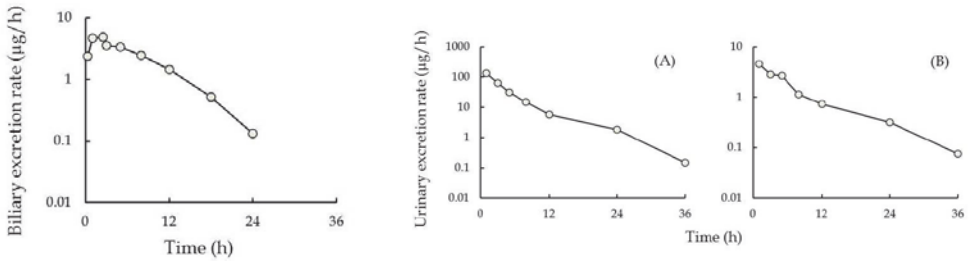
PI polyamide A

Parameter	Dose			
	1.3 mg/kg	2.0 mg/kg	7.5 mg/kg	15.0 mg/kg
Body weight (kg)	0.267	0.291	0.243	0.26
$t_{1/2}$ (min)	54.8	42.3	74.8	45.3
C_0 (μg/mL)	14.1	22.9	77.1	227.5
AUC (μg min/kg)	259.6	316.8	1528.6	3331.9
Cl (mL/min/kg)	5.6	6.4	5.1	4.6
Vss (mL/kg)	305.8	274.6	411.8	243.7
MRT (min)	68.1	42.6	80.5	54

PI polyamide B

Parameter	Dose			
	1.0 mg/kg	2.0 mg/kg	3.0 mg/kg	5.0 mg/kg
Body weight (kg)	0.313	0.317	0.317	0.317
$t_{1/2}$ (min)	139.1	165.8	207.3	359.3
C_0 (μg/mL)	1.5	4	3.8	4
AUC (μg min/kg)	108.1	205.2	326.8	508.3
Cl (mL/min/kg)	9.9	8.9	9.2	10.3
Vss (mL/kg)	2170.5	1990.1	2602.2	4567
MRT (min)	194.7	222.5	289.7	492.1

Biliary and urinary excretions of Polyamides



The biliary excretion rate of PI polyamide A showed saturation at the early period, while PI polyamide B was not detected in the bile. The urinary excretion rates of PI polyamides A and B showed a linear elimination. The cumulative urinary excretion rates of PI polyamides A and B at 48 h were 72 and 5 % of the administered dose. The cumulative biliary excretion rate of PI polyamide A at 24 h was 4 %. These observations indicated that unchanged PI polyamides A and B were slowly eliminated from the body (Nagashima et al. 2010).

Safety study of PI polyamide（Lethal dose & side effects）

Administration of PI polyamide into Mice

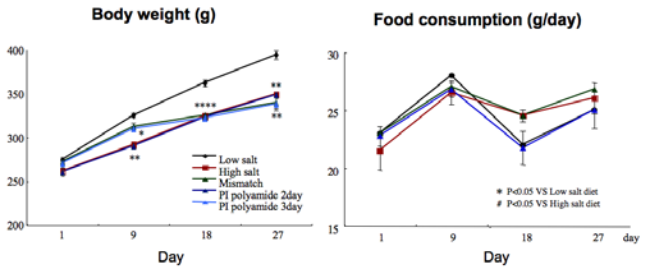
Classification	Weight (g)	Conc. (mg/mL)	Dose (mg/kg)	Result
High dose	32.15	14.26	79.84	Dead
High dose	30.88	14.26	92.36	Dead
Low dose	32.92	7.13	43.32	Dead
Low dose	32.21	7.13	22.14	Alive

Changes in BW; Day 0: 32.2 g, Day 2: 31.7 g, Day 3: 31.5 g, Day 4: 32.3 g, Day 5: 32.5 g

Five days after administration of PI polyamide, mice were scrified and plasma data were analysed. Levels of total protein, A/G ratio, total cholesterol, urea nitrogen were normal. AST was relatively high, ALT was in normal range, and ALP was same as control. These data indicate that the lethal doses of polyamide are over 40 mg/kg, under 20 mg/kg is safety dose, and PI polyamides do not show obvious side effects in mice.

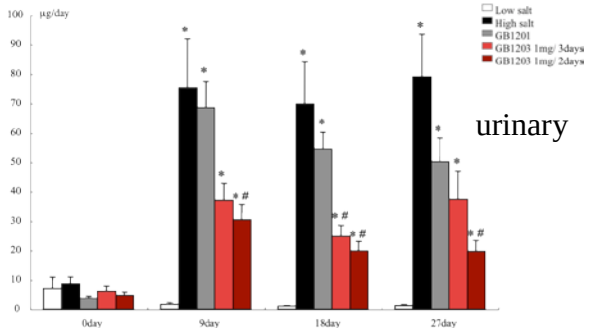
Long term effects of PI polyamide targeting TGF-β1 on progressive renal diseases

We investigated the long term effects of 1 mg/kg of PI polyamides to TGF-β1 in Dahl S-rats for 4 weeks. Body weight and food consumption were not affected with the long term administration. These data indicate that PI polyamides have no systematic side effects (Matsuda et al. 2011).



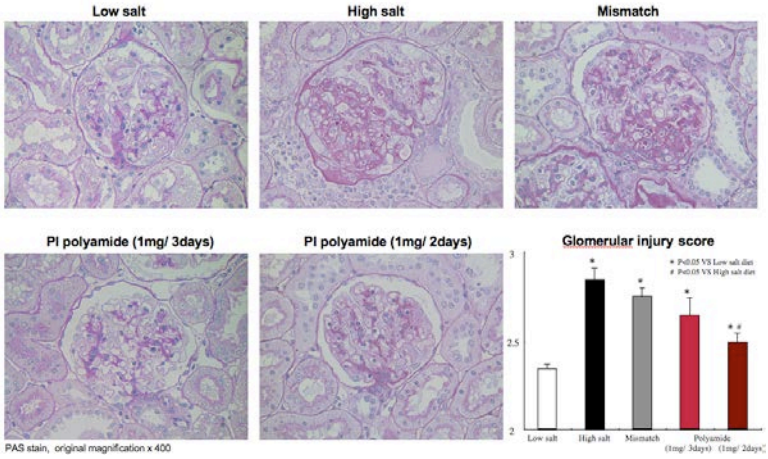
Effects of PI polyamide targeting TGF-β1 on urinary excretion of TGF-β1

Sysntematic administration of PI polyamide targeting TGF-β1 significantly decreased excresion of TGF-β1 for 4 weeks in salt-loaded Dhal-S rats (Matsuda et al. 2011).



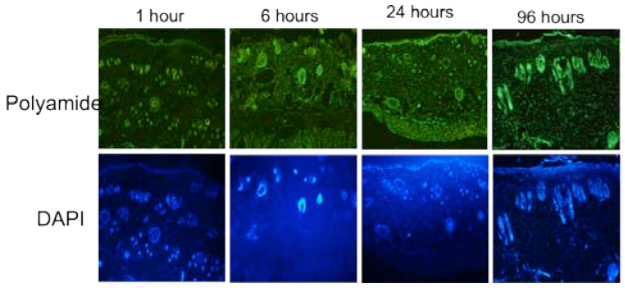
urinary

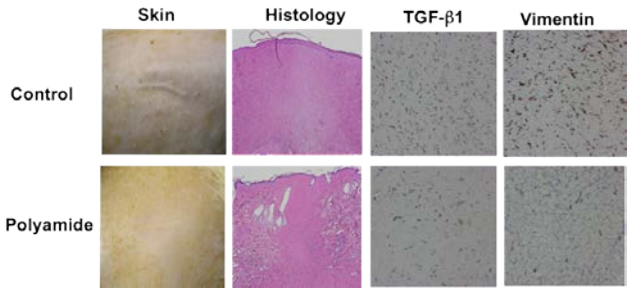
Effects of the long term administration of PI polyamide to TGF-β1on glomerular injury in Dhal-S rats after 4 weeks. PI polyamide to TGF-β1 significantly improved glomerular injury.



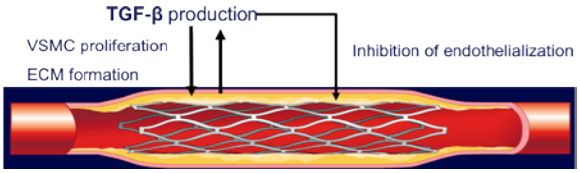
Effects of PI polyamide targeting TGF-β1 on skin hypertrophic scar (Washio et al. 2010)

FITC-labeled TGF-β1-targeted PI polyamide was distributed in nuclei of almost all cells in incisional skin wounds as shown in right figure. Even at 1 hour after a singledose subcutaneous injection, FITC-labeled Polyamide was strongly localized in the nuclei of keratinocytes in the epidermis and inflammatory cells, fibroblasts, and hair follicle cells in the dermis. Right figure shows effects of Polyamide on development of hypertrophic scars. Injection of Polyamide completely inhibited the hypertrophic scars. Histological findings also showed that Polyamide markedly inhibited fibrotic degeneration of incisional wounds compared with control. Injection of Polyamide reduced TGF-β1 staining and the number of vimentin positive fibroblasts.



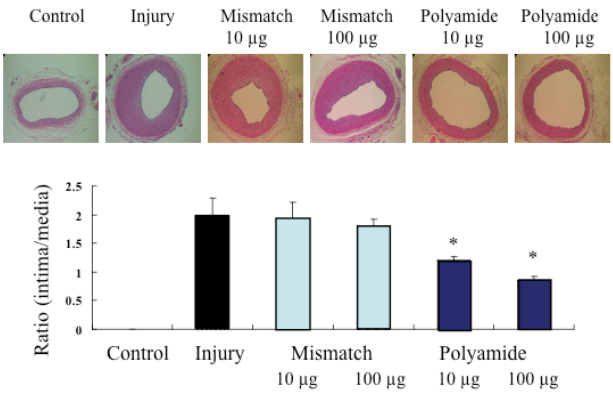


PI polyamide targeting TGF-β1 inhibits restenosis and preserves endothelialization in the injured artery (Yao et al. 2009)

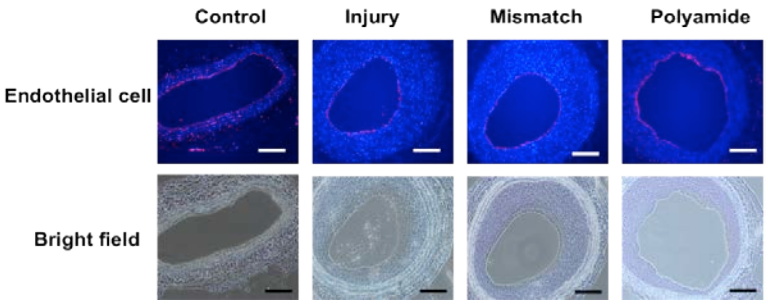


TGF-β1 plays an important role in the pathogenesis of restenosis. TGF-β1 stimulates proliferation of VSMC which show the synthetic phenotype. TGF-β1 inhibits the endotheliarization in stents. TGF-β1 increases the neointima with ECM formation.

Right figures show the effects of PI polyamide targeting TGF-β1 on neointimal formation in rat carotid artery at 21days after balloon injury. Both 10 and 100 μg of PI polyamides significantly reduced neointimal thickening by 39 and 57%, respectively, when compared with the injury group. The mismatch polyamide did not affect neointimal thickening.



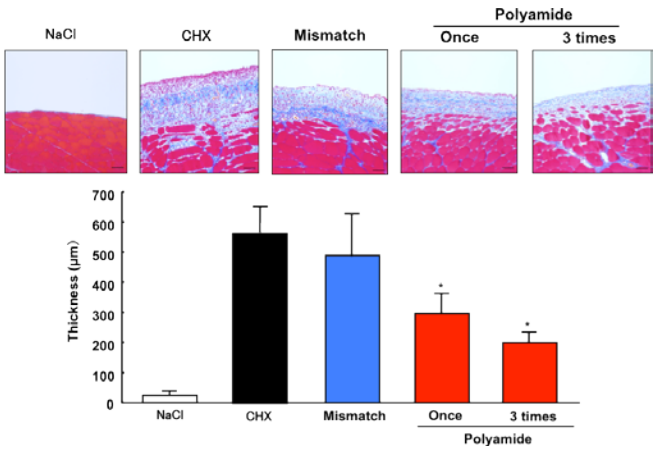
Right figure shows the immunohistochemistry findings of ECs with the anti-von Willebrand factor antibody in rat carotid artery at 21 days after balloon injury. ECs were stained in the intimal surface after balloon injury. The treatment of PI polyamide to TGF-β1 obviously enhanced the staining of ECs in the injured artery.



PI polyamide targeting TGF-β1 will be a potentially effective agent for the treatment of in-stent restenosis, as a candidate agent for the next-generation of the drug-eluting stent.

Effects of PI polyamide to TGF-β1 on thickning of peritoneum in encapsulating peritoneal sclerosis (EPS) model rats (Serie et al. 2011).

One mg of Polyamide i.p. injected once or 3 times in rat models given a daily i.p. injection of chlorhexidine gluconate and ethanolfor 14 days significantly suppressed the thickness of the peritoneum (right figure). These data suggest that PI polyamide targeted to the TGF-β1 promoter will be a specific and feasible therapeutic strategy for patients with EPS.



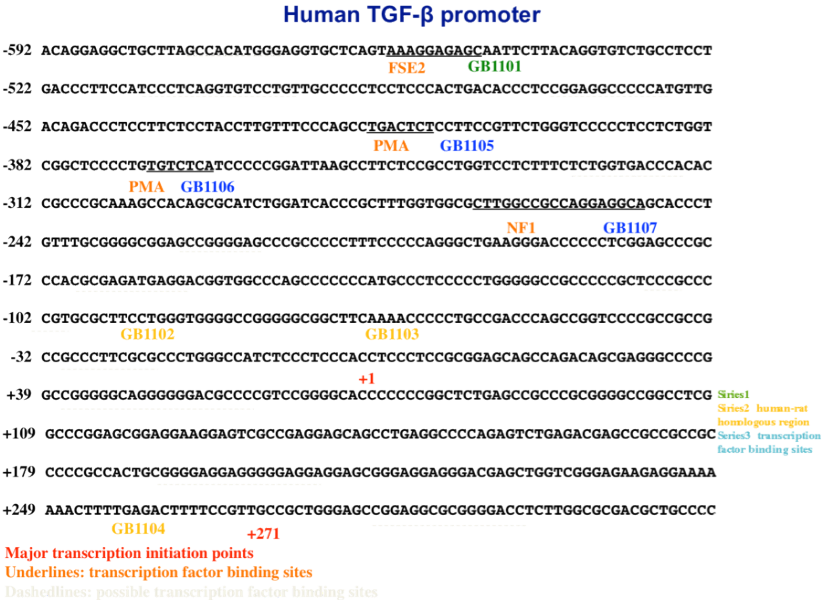
Specificity of PI polyamide to TGF-β1 to TGF-β1 gene in kidney from salt loaded-Dahl-S rats

To evaluate specificity of PI polyamide to TGF-β1, we determined expression of over 30,000 transcripts by microarrays of the renal cortex following treatment with Polyamide. Polyamide down-regulated 9 out of 681 growth factor transcripts, 11 out of 685 cytokine transcripts, and 8 out of 397 ECM transcripts. We searched for AP-1 binding sites on about 3000 nucleotides upstream of each genomic region with TFSEACH (http://mbs.cbrc.jp/research/db/TFSEARCH.html). Among these down-regulated genes, four genes had common sites of AP-1 binding and possible Polyamide binding sites. These data indicate PI polyamide to TGF-β1 specifically inhibits mRNA expression of TGF-β1 without the off targeting effects (Matsuda et al. 2011).

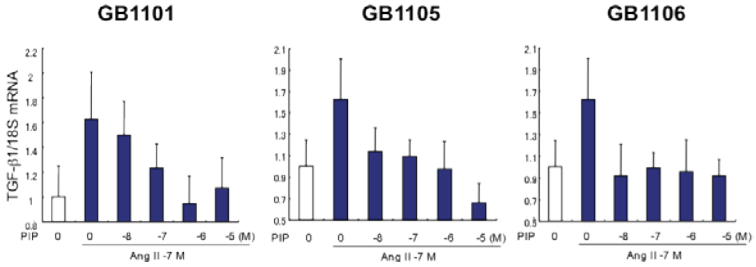
Suppressed genes 14/681 whole growth factor genes

AC#	Gene name	FD	AP-1	PI polyamid2	AP-1 and PI polyamide2
BC076380	transforming growth factor β-1	0.542	2	9	1
AA955871	Insulin-like growth factor 1	0.531	1	11	0
BC083173	activin A receptor type II-like 1	0.528	2	14	0
AF140232	S100 calcium binding protein A6 (calcyclin)	0.525	2	14	0
NM_017123	amphiregulin	0.516	3	16	0
AI598730	neurotrophin receptor associated death domain	0.482	6	12	0
BM391471	activating transcription factor 5	0.481	3	16	0
BI290885	follistatin-like 1	0.476	3	12	0
NM_053887	mitogen activated protein kinase kinase kinase 1	0.475	3	14	1
NM_021586	latent transforming growth factor beta binding protein 2	0.431	1	17	0
AA859669	neuropilin 2	0.398	1	15	0
BE099439	Kazal-type serine peptidase inhibitor domain 1	0.396	5	18	1
J02941	renin 1	0.356	0	8	0
BG379319	transforming growth factor β induced	0.237	0	0	0

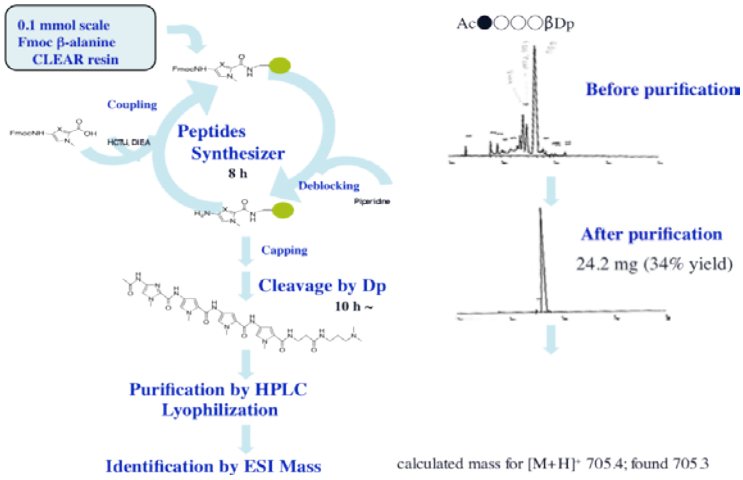
Lead optimization of PI polyamides targeting human TGF-β1



To determine a lead compound of PI polyamides targeting human TGF-β1, we firstly analyzed the promoter stucture of human TGF-β1 gene, and examined effects of the PI polyamides on angiotensin II-stimulated expression of TGF-β1. Then GB1101, GB1105, and GB1106 are potential candidates for the lead compound of PI polyamides targeting human TGF-β1.



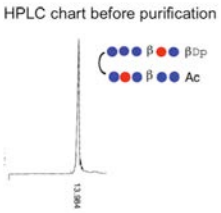
Development of large scale synthesis of PI polyamide



Example of automatic synthesis procedure

Using the peptide synthesizer PSSM-8 fromShimazu, Co.Ltd., over 1000 mg of polyamide can be synthesized automatically now. We are developing more large scale industrial synthetic technology as 100 g once in GMP grade.

Shimazu, Co.Ltd



Patents for PI polyamides by Gentier Biosystems Inc.

1. PI polyamides targeting human TGF-β1
   (PCT/JP2008/014079, WO2006/018967, US7888516: Feb.15, 2011)
   http://www.google.com/patents/about/11_658_475_Tgf_ß_Gene_Expression_Inhibi.html?id=4WyuAAAAEBAJ
   Inventors: Noboru Fukuda, Takahiro Ueno
   Original Assignees: Nihon University, Gentier Biosystems Incorporation
   Abstract: A TGF-β gene expression inhibitor containing a pyrrole-immidazole polyamide comprising an N-methylpyrrole unit (hereinafter also referred to as Py), an N-methylimidazole unit (hereinafter also referred to as Im) and a γ-aminobutyric acid unit which can be folded into an U-shaped conformation at the above-described γ-aminobutyric acid unit site in a minor groove of a double-stranded region (hereinafter referred to as the target region) containing a part or the whole of the following base sequence corresponding to −450 to −310 of human transforming growth factor β1 (hereinafter also referred to as h TGF-β1) promoter and a strand complementary thereto and in which a Py/Im pair, an Im/Py pair and a PY/Py pair correspond respectively to a C-G base pair, a G-C base pair and an A-T base pair and a T-A base pair.

2. PI polyamides targeting human CTGF
   (PCT/JP2006/318512, WO2007/034784)
   http://www.wipo.int/patentscope/search/en/WO2007034784
   Inventors: Noboru Fukuda, Takahiro Ueno, Hiroshi Sugiyama
   Original Assignees: Nihon University, Gentier Biosystems Incorporation

Abstract: Disclosed is an inhibitor of the expression of CTGF gene, which comprises a pyrrole-imidazole polyamide. The pyrrole-imidazole polyamide has an N-methylpyrrole unit (hereinafter, referred to as “Py”), an N-methylimidazole unit (hereinafter, referred to as “Im”) and a ƒ-aminobutanoic acid unit. In the pyrrole-imidazole polyamide, the region of the ƒ-aminobutanoic acid unit is folded to form an U-shaped conformation in a miner groove of a double-helical region having a part or the entirety of a nucleotide sequence lying between position -195 to position -150 (SEQ ID NO:2) in a connective tissue growth factor (hereinafter, referred to as “CTGF”) promoter or a sequence complementary to the nucleotide sequence (hereinafter, the double-helical region is referred to a 'target region'). In the pyrrole-imidazole polyamide, a Pm/Im pair corresponds to a C-G base pair, an Im/Py pair corresponds to a G-C base pair, and a Py/Py pair corresponds to both an A-T base pair and a T-A base pair.

3. PI polyamides targeted to human LOX-1 gene (JP 2006-022063)
http://jglobal.jst.go.jp/detail.php?JGLOBAL_ID=200903003017803187&q=&t=2 Gene silencing agent coronary atherosclerosis and stenosis for the drug-eluting stent

4. Automated solid-phase synthesis of PI polyamides
(PCT/JP2006/322658, WO2007/060860**)**
http://patent.ipexl.com/WO/2007ZZSLASHZZ060860.html
Inventors: Noboru Fukuda, Syuzei Dohno, Hiroshi Sugiyama
Original Assignees: Nihon University, Gentier Biosystems Incorporation
Abstract: A synthesis method for PI polyamide is automated at a higher level and can produce a product with a high yield in a more stable manner. In a solid phase synthesis method, the automated synthesis of a polyimide can be promoted by using HCTU as a condensation-activator, the yield of a product can be increased by charging a monomer in a solid form, and a PI polyamide having any sequence can be synthesis by combining the synthesis method with a manual synthesis with an acid chloride.

Compatible patents

1. Complex formation between dsDNA and oligomer of cyclic heterocycles
US6472537B1 http://www.patentstorm.us/patents/6303312.html
Inventors: Peter B. Dervan, Joel M. Gottesfeld

Assignee: California Institute of Technology

Abstract: Methods and compositions are provided for forming complexes intracellularly between dsDNA and oligomers of heterocycles, aliphatic amino acids, particularly omega-amino acids, and a polar end group. By appropriate choice of target sequences and composition of the oligomers, complexes are obtained with low dissociation constants. The formation of complexes can be used for modifying the phenotype of cells, either prokaryotic or eukaryotic, for research and therapy.

Influences to our patents: This patent is the general patent for PI polyamides as siRNA and antisense. Our patents are specific for the compounds, which have priolity to the general patents. Dervan's patents will be extinction of right at 2017.

2. Design, synthesis and use of specific polyamide DNA-binding ligands

http://www.freepatentsonline.com/7087378.html

Inventors: Baird, Eldon E., Dervan, Peter B.

Assignee: California Institute of Technology

Abstract: The invention encompasses improved selective polyamides for binding to specific nucleotide sequences of double stranded DNA as well as methods for designing and synthesizing polyamide DNA binding ligands that are selective for an identified specific nucleotide sequence. The 3-hydroxy-N-methylpyrrole/N-methylpyrrole carboxamide pair specifically recognizes the T•A base pair, while the N-methylpyrrole/3-hydroxy-N-methylpyrrole pair recognizes A•T nucleotide pairs. Similarly, an N-methylimidizole/N-methylpyrrole carboxamide pair specifically recognizes the G•C nucleotide pair, and the N-methylpyrrole/N-methylimidizole carboxamide pair recognizes the C•G nucleotide pair.

Influences to our patents: Our patent for the synthesis of PI polyamides are by the automatic solid phase methods using HCTU as a coupling reagent, which are completely different from Dervan's patent.

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