DOI: 10.1002/cmdc.200((will be filled in by the editorial staff))

Chemically Modified Synthetic Small Molecule Boosts its Biological Efficacy against Pluripotency Genes in Mouse Fibroblast

Abhijit Saha,[a] Ganesh N. Pandian,[b] Shinsuke Sato,[b] Junichi Taniguchi, [a] Yusuke Kawamoto,[a] Kaori Hashiya,[a] Toshikazu Bando,*[a] and Hiroshi Sugiyama*[a],[b],[c]

*Small molecules capable of inducing targeted transcriptional activation have immense opportunities in cellular reprogramming. Previously, we identified a synthetic transcriptional activator encompassing both sequence-specific pyrrole-imidazole polyamides (PIPs) and epigenetic activator (SAHA) **1** that can dramatically induce the endogenous expression of core pluripotency genes. Extensive microarray data analysis revealed that Oct-3/4 as the target pathway of **1** but not SAHA. However, the expression levels in MEFs treated by **1** were relatively lesser than that in mouse embryonic stem (ES) cells. In this study we tried to improve its biological efficacy by employing carbohydrate solubilizing agent 2-hydroxypropyl-β cyclodextrin (HpβCD) in the cell culture medium. Although HpβCD increased the solubility of **1** under in vitro conditions, biological efficacy of **1** could not be notably improved with HpβCD treatment. In addition a C-terminus modification of **1** through incorporation of an isophthalic acid (IPA) increases its biological efficacy significantly. Here, we report for the first time about the development of a mild convergent synthetic procedure to incorporate IPA at the C-terminus of the PIP to achieve **2**, an innovative small molecule capable of inducing Oct-3/4 with the expression levels comparable to that in ES cells.*

Introduction

Using combination of seven small molecules Deng and coworker artificially induced pluripotency in mouse somatic cells.[1] Since this transgene free approach enhances the clinical prospects of induced pluripotent stem (iPS) cell technology, screening and identification of small molecules that could induce the pluripotency genes are now in rising demand.[2a] Several chemical modulators of epigenetic enzymes and/or signalling pathway factors have shown success in enhancing the somatic cell reprogramming.[2b] However, the lack of selectivity and requirement of multiple small molecules is a major concern. In this regard, we previously conjugated the histone deacetylase inhibitor SAHA (suberoylanilide hydroxamic acid) with hairpin pyrrole-imidazole polyamide (PIPs) to create a new class of small molecules called SAHA-PIP.[3] PIPs are sequence specific DNA minor groove binding small molecules having the binding ability similar to natural transcriptional binding proteins.[4, 5] Sequence selectivity of these PIPs is programmed by the side by side stacked ring pairing such as I/P distinguishes G.C from C.G and P/P to either A.T or T.A.[6] Hence, distinctive PIPs may direct SAHA and induce differential transcriptional activation.[7,8] Screening studies carried out for evaluating the effect of 32 distinct SAHA-PIPs on pluripotency genes in mouse embryonic fibroblasts (MEFs) revealed that certain SAHA-PIPs could differentially induce Oct-3/4, Sox2, Klf4 and c-Myc.[8] In particular, a SAHA-PIP **1** (Figure 1), targeting 5'-WGWWC-3' (W=A/T) sequence could rapidly induced multiple pluripotency genes in just 24 h with 100 nM treatment.[9,10] Extensive analysis of the microarray data on genome-wide gene expression in MEFs revealed that **1** significantly induce Oct-3/4 pathway genes that are related to embryonic stem cell pluripotency.[10] None of the top ten significantly enhanced pathways in SAHA treated MEF were related to embryonic stem cell pluripotency. Although, **1** could notably upregulate pluripotency genes in MEFs, the expression levels were still lower when compared to that in ES cells.

It is important to consider that cellular uptake of PIPs hinders its endogenous gene expression.[11] A general hypothesis for polyamide biological activity relied on its cell uptake and competing efflux pathway influences its accumulation in cell nucleus. Increasing the biological activity of PIP may infer a decrease in efflux.[12] Here in this report we tried to improve biological activity of **1** by employing carbohydrate solubilizing agent such as 2-hydroxypropyl-β-cyclodextrin (HpβCD) in cell culture medium. It has been found that polyamide forms

[a] *A. Saha, J. Taniguchi, Y. Kawamoto, K. Hashiya, Dr. T. Bando, Prof. Dr. H. Sugiyama*
Department of Science, Graduate School of Science, Kyoto University, Sakyo, Kyoto 606-8501 (Japan).
Fax: (+)81-75-753-3670
E-mail: hs@kuchem.kyoto-u.ac.jp
[b] *Dr. G. N. Pandian, S. Sato and Prof. Dr. H. Suigiyama*
Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Yoshida Ushinomiya-cho, Sakyo, Kyoto 606-8502.
[c] *Prof. Dr. H. Sugiyama. CREST, Japan Science and Technology Corporation (JST),Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan*

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author.

measurable particle size or aggregates under biologically relevant conditions.[12] Hence we decided to check the ability of HpβCD to

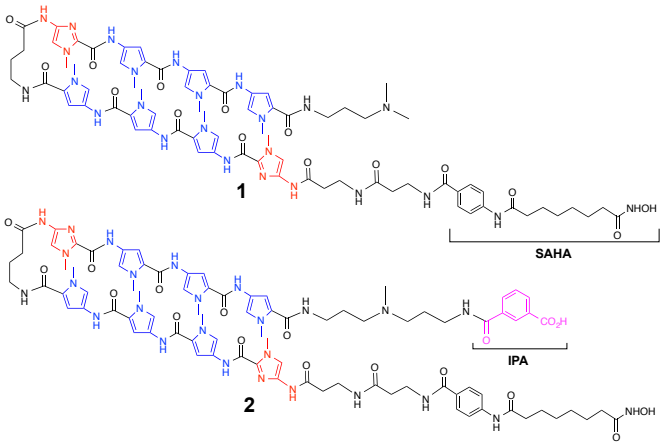


Figure 1. Chemical structures of **1** and its C-terminus modified analogue as **2**.

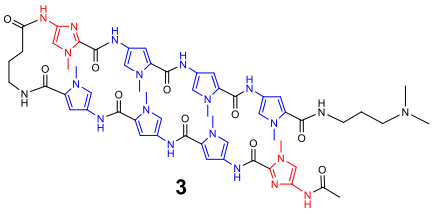
decrease aggregation of **1**. We also focused on structural modification of **1** at its C-terminus end. A varied structural motif change at the C-terminus of a polyamide has been studied by Dervan and co-workers and among many choices isophthalic acid (IPA) incorporation enhances its solubility.[11] Thus, we demonstrate to synthesize IPA-conjugate **2** to incorporate in SAHA-PIP **1**, and evaluate its gene-activating activity.

Results and Discussion

2.1. Solubility 1 with or without CD using HPLC

Increasing the solubility of **1** could improve its biological efficacy as cellular uptake and nuclear localization often hinder the PIP mediated endogenous gene regulation. Employment of employing 2-hydroxypropyl-β-cyclodextrin (HpβCD) was known to increase the solubility of PIP. In order to determine the effect of HpβCD on SAHA-PIP, first we checked the solubilities in presence and absence of cyclodextrin. The macroscopic solubility were investigated by measuring the HPLC peak area, detected at 254 nm, of the respective polyamide solution (same concentration) in the presence of 0-50mM HpβCD and compared with the peak area of the same polyamide dissolved in DMSO, considered to be 100% solubility.

For each polyamide two kinds of solubility were measured; one in the presence of HpβCD (+) and another in absence of it (-). Conjugate **3** which contains an acetyl cap at the N-terminus showed enhancement in solubility in the presence of CD (+) whereas in absence of it (-) solubility is less. Whereas conjugate **1** which have β-Ala-β-Ala-SAHA at the N-terminus showed very poor solubility in absence of CD (-); however its solubility increased dramatically in the presence of CD (+). This implies probable aggregation or precipitation occurs in both **1** and **3** under biological relevant conditions. This result suggests the presence of ββSAHA at the N-terminus more likely increases aggregation propensity in SAHA-PIPs.



solubility	
1 CD (-)	: 3-4%
1 CD (+)	: 31-36%
3 CD (-)	: 25-41%
3 CD (+)	: 51-54%

Figure 2. Calculated solubility of polyamide **1** and **3** in 0.1% DMSO/PBS containing 0-50 mM HpβCD at room temperature. Resultant solubility was determined by HPLC peak area at 254 nm detection. 100% solubility was calculated from the peak area of the respective polyamide dissolved only in DMSO.

2.2. Effect of CD in MEF treated by 1

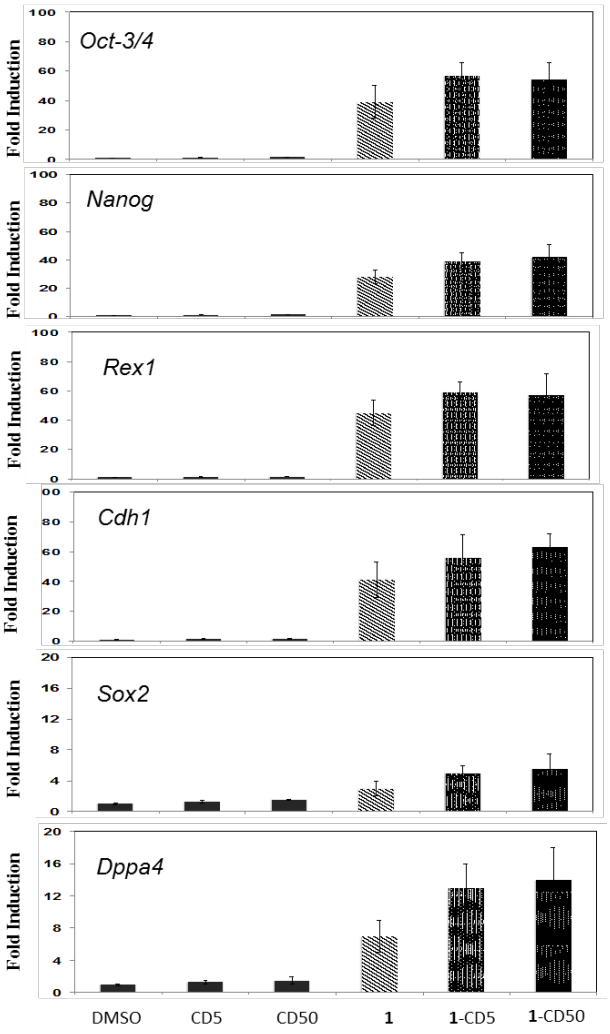


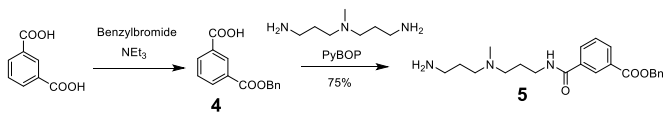
Figure 3. Effect of HpβCD (5 mM and 50 mM) on **1** on the endogenous expression of pluripotency genes. Expression levels of Oct3/4, Nanog, Rex1, Cdh1, Sox2, and Dppa4 were determined by treating MEF with 100 nM of **1** to each sample, and with 0.1% DMSO, 5 mM and 50 mM of CD as control experiment. Each bar represents the mean ± SD from 18 well plates.

Based on the previous results, we tried to overcome the challenge of solubility by employing HpβCD in cell culture. Quantitative real time polymerase chain reaction (qRT-PCR) can

be used as technique to check cellular uptake as measurement of the endogenous mRNA levels (here Oct-3/4 pathway genes) could be inferred as a biological readout of the effectors. CD (5 mM or 50 mM) itself did not modulate the core pluripotency gene expression. Treatment of **1** dissolved in either 5 mM or 50 mM of HpβCD also showed only a marginal (a maximum of about 1.5 fold) increase in the endogenous expression of the core pluripotency genes (Oct-3/4, Nanog, Cdh1, Rex1, Sox2 and Dppa4) (Fig. 3). Since the induction values are still within the expression levels of **1**, the effect of CD vehicle is negligible. Hence, an alternative strategy is required to improve the biological efficacy of **1**.

3. Synthesis of Isophthalic acid (IPA) tail and 2

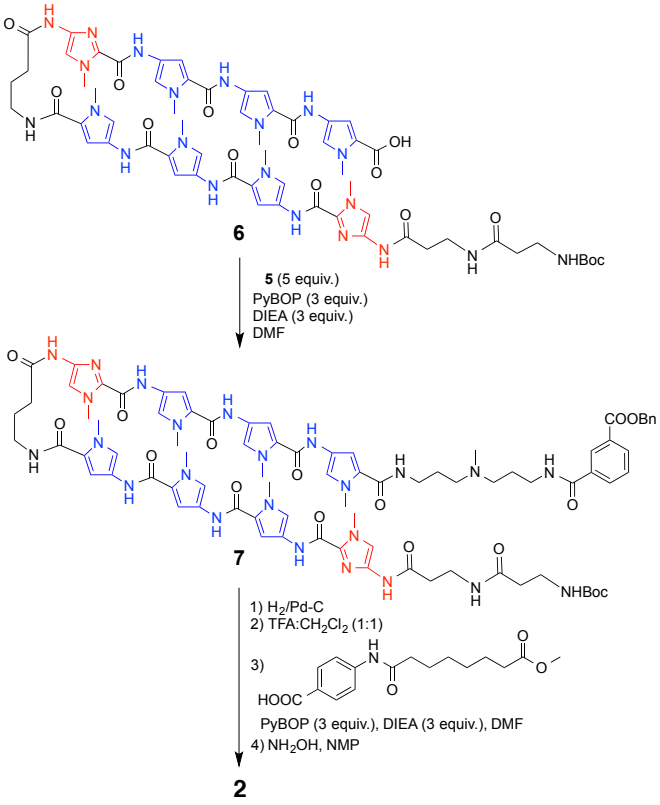
Previous gene regulation studies in cell culture relied on the results obtained with fluorescein labelled PIPs as a cell permeable small molecule.[13] In recent years, a non-fluorescent agent like isophthalic acid (IPA) at the C-terminus of a polyamide has substituted the fluorescent tag and rivalled the activity of original FITC labelled polyamide.[11,14] Therefore we thought to incorporate an IPA at the C-terminus of the **1** to check its biological activity. However, the synthesis scheme of IPA conjugated SAHA-PIP is difficult with the existing time-consuming synthetic route. Hence, a milder approach to achieve such compound in good yield is warranted. Herein, for the first time we report the synthesis of IPA conjugated **1**, termed **2**. We incorporated an isophthalic acid (IPA) at the C-terminus of **1** by first making an IPA tail (Scheme 1) and then coupled it with COOH group present at the C-terminus of the polyamide as shown in scheme 2.



Scheme 1. Synthesis of C-terminus-IPA tail.

The synthesis of IPA tail started with the monoprotection of isophthalic acid (IPA)[15] using benzyl bromide and then we tried mono protection of 3, 3-diamine N-methyldipropylamine (triamine). In previous Dervan`s protocol they synthesized mono boc protected triamine to synthesize the IPA tail.[16] However the synthesis is difficult as we tried the mono boc protection of the triamine but a large amount of diprotection occurred. Then without monoprotection of the triamine, we tried the coupling with mono protected IPA using PyBOP. Within 1h, the reaction underwent smoothly and obtained mixer of two products having mono and di coupling product in the ratio of 5:1 respectively in HPLC profile (Scheme 1).

The long polyamide conjugate (boc-β-β-Im-Py-Py-Py-γ-Im-Py-Py-Py-COOH) **6**, was synthesized using Fmoc solid phase chemistry by PSSM-8 peptide synthesizer (Shimadzu, Kyoto) with computer assisted operating system. One pyrrole loaded oxime resin 45 mg (ca. 0.2 mmol/g, 200-400 mesh) was used to start the synthesis in the solid phase machine. Fmoc unit (such as Fmoc-Py-COOH, Fmoc-Im-COOH or Fmoc-β-Ala-OH) (0.20 mmol)used in each step, were dissolved in NMP. The condition in each cycle of solid phase synthesis were: twice deblocking with 20 % piperidine/DMF (0.6 mL) for 4 min, preactivation of –COOH group for 2 min with HCTU (88 mg, 0.21 mmol) in DMF (1 mL) and 10% DIEA/DMF (0.4 mL), coupling time for 1 h and washing with DMF. After SPPS, the polyamide was detached from resin using 2M NaOH (aq.) in dioxane at 55 °C for 3h (Scheme 2). The reaction mixture was filtered and washed with Et_2O, to yield a crude yellow powder. Without further purification the precursor **6** (20 mg) was dissolved in DMF (0.5 mL) and coupled with preassembled IPA-tail (5) using PyBOP and DIEA. This allowed the formation of precursor **7** containing C-terminus IPA-tail. The reaction was quenched by adding water and then washed with Et2O several times to achieve powder. The crude **7** without further purification was reduced using H2/Pd-C and followed by boc deprotection at the N-terminus using TFA. Excess TFA was removed by vacuum pump and the crude reaction mixture was washed with Et_2O which yield crude gray powder. In the next step, the polyamide having free amine at N-terminus was coupled with HOOC-SAHA-(Ome) using PyBOP and DIEA. This reaction allowed the formation of methylester of **2**. In the final step methyl ester of SAHA converted to the active NHOH, which allowed the final product **2,** by performing the reaction in aqueous solution of 50% (v/v) NH_2OH (0.5 mL).



Scheme 2. Design and synthesis of isophthalic acid (IPA) conjugated SAHA-PIP **2**

4. The Effect of 2 in MEF

The purified **1** and **2** were then treated with MEF at the concentration of 100 nM and effect of these two effectors was then evaluated as mentioned before.[10] Interestingly, **2** induced the endogenous expression of Oct-3/4 and Nanog by about 80-fold and 60-fold, respectively (Fig. 4a and 4b). These induction

values were notably higher than that observed in MEFs treated by **1** and were also comparable to the pattern observed in ES cells (Fig. 4a and 4b). Interestingly, about 100-fold increase in the endogenous expression of Rex1, a critical pluripotency gene that is typically silenced in somatic cells like MEFs was observed with just 24h treatment of **2** (Fig. 4c). Also, **2** notably induced Cdh1, a critical mesenchymal epithelial transition (MET) marker by about 100-fold (Fig. 4d). On the other hand, the unmodified **1** induced Rex1 and Cdh1 by about 40-fold (Fig. 4c and 4d). Although, **2** biological activity appears to be superior to **1**, the induction values is still low when compared to that observed in ES cells ((Fig. 4c and 4d). However, this result also suggests the possibility of Oct-3/4 to be the potential target gene of **1**. Similar pattern was observed in the expression profile of Sox2 and Dppa4 (Fig. 4e and 4f). It is important to note here that we previously demonstrated that SAHA alone had no effect on any of these pluripotency genes. Interestingly, **2** mildly increased the expression of even Klf4 and c-Myc (data not shown), the genes known to follow a different pathway. The dramatic enhancement of Cdh1 could be the reason behind such bioactivity of **2**. The relevance of this current study will provide a vital troubleshooter during the scheme of increase in number of base pair recognition of this polyamide for more sequence specificity.

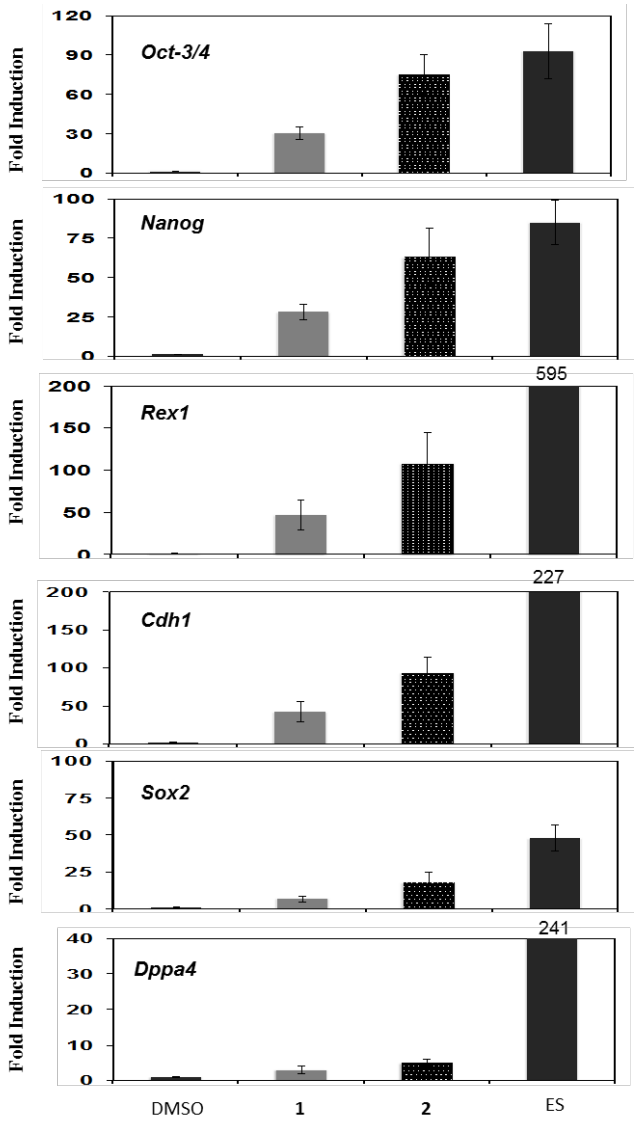


Figure 4. Effect of **2** on the endogenous expression of pluripotency genes. Expression levels of Oct3/4, Nanog, Rex1, Cdh1, Sox2, and Dppa4 were determined by treating MEF with 100 nM of **1** and **2** individually, and with 0.1% DMSO as a control. ES cells were used as positive control. Each bar represents the mean ± SD from 18 well plates.

Artificial induction of pluripotency state in a somatic cell by small molecules proposes various applications in regenerative medicine. In this regard, discovery of **2**, the first ever small molecule capable of inducing critical developmental genes like Oct3/4 and Nanog, Rex1 and Cdh1 by about 100-fold in MEFs, could lead to paradigm-shifting opportunities in cellular reprogramming. In recent years, many groups including our group have extensively studied the issue of polyamide solubility. As a result many new developmental solutions have been taken to enhance the cellular uptake such as pyrrol/imidazole content,[17] C-terminus modification with isophthalic acid,[11] presence of β-aryl turn at the γ-aminobutyric acid turn of a hairpin polyamide[18] etc. In particular, designing of PIPs having more number of DNA base pair recognition ability would be hindered due to their poor cellular uptake. SAHA-PIP **1** having 6 base pairs DNA recognition sequence can switch `ON` the silent pluripotency gene network, however their efficiency was lower than that observed in ES cells. It might be due to the efflux of PIP and because sufficient number could not reach the target position where it could bind at the promoter region of gene and activate the transcription factor. Therefore, we thought that the incorporation of IPA with SAHA conjugates could improve its biological efficacy and this effect could be measured using qRT-PCR. Accordingly, in almost all genes tested we noticed a significant improvement about 3 fold in some gene expression after IPA incorporation. Recently certain SAHA-PIPs were shown to distinctively activate developmental genes in human dermal fibroblasts. The novel synthetic route described in this report to achieve PIP-IPA would be helpful for our future studies in particular increasing the sequence recognition ability by increasing the number of base pairs in SAHA-PIP. Although, longer SAHA-PIP would be more specific to target sequences, they are also known to have poor cellular uptake. We propose that we could overcome such challenge through IPA incorporation.

Conclusion

In summary we studied cellular uptake by **1** in cell culture medium. We used carbohydrate formulating agent cyclodextrin to prevent aggregation of the polyamide to increase its biological activity. It was found that solubility of **1** in presence of cyclodextrin increased significantly in vitro; however a marginal enhancement noticed about 1.5 fold in endogenous gene expression. Therefore an alternative approach to improve cell uptake of **1** needed. By structural modification of **1** such as incorporation of an isophthalic acid (IPA) at its C-terminus this could be achieved. Here we have designed and synthesized a SAHA-PIP with improved cell uptake. In our new synthetic procedure we overcome the low yield of the existing protocol and generate a PIP-IPA conjugate with high purity. Introduction of IPA in SAHA-PIP showed increased potency in pluripotency gene expression about 3 fold which immediately useful for testing in long term treatment, where the ability to work at less con-centration of polyamide will help to overcome challenges regarding solubility of polyamide and

formulation. The amenability of IPA conjugation at the C-terminus of SAHA-PIP also raises many possibilities to improve further by putting a β-aryl turn at the γ-aminobutyric acid turn of SAHA hairpin polyamide.

Experimental Section

General

All reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations used here: Py, N-methylpyrrole; Im, N-methylimidazole; Fmoc, 9-fluorenylmethoxycarbonyl; HCTU, 1-[bis(dimethylamino)methylene]-5-chloro-1H-benzothiazolium 3-oxide hexafluorophosphate; DMSO, dimethylsulfoxide; DMF, dimethylformamide; DIEA, N,N-diisopropylethylamine; β-alanine; γ-aminobutyric acid; SAHA, suberoylanilide hydroxamic acid, HpβCD, 2-hydroxypropyl- ***β*** -cyclodextrin; Pd-C, Palladium on carbon (10 % of loading) and Dp, N, N-dimethylaminopropylamine. ^{1}H NMR spectra were recorded on a JEOL JNM-FX 600 model NMR spectrometer. HPLC analysis was performed with a 4.6 x 150 mm column on a JASCO PU-2089 plus pump model with UV-2075 plus HPLC UV/VIS detector and a Chemcobond 5-ODS-H 10 x 150 mm column (Chemco Scientific Co., Ltd, Osaka, Japan) was used for the purification of polyamide conjugates. Electrospray Ionization Time-of-Flight mass (ESI-TOF-MS) was recorded on BioTOF II ESI-TOF Bruker Daltonics Mass Spectrometer (Bremen, Germany). Flash column system was performed using Combi Flash Companion model (Teledyne Isco Inc., NE, USA).

Synthesis of SAHA-β-β-Im-Py-Py-Py-γ-Im-Py-Py-Py-Dp, (1). Using solid phase peptide synthesizer conjugate **1** was synthesised. After SPPS, it was detached from the resin using N, N-dimethylaminopropylamine (Dp), stirred at 45 ^{o}C for 3h. The reaction mixture was filtered, triturated from Et_2O, to yield as a yellow crude powder (21 mg). Without further purification the precursor was dissolved in 150 μL of NMP and later another 150 μL of aqueous solution of 50% (v/v) NH_2OH were added, to convert methylester of SAHA to the active NHOH, this allows the formation of **1**. The reaction was quenched by adding 150 μL of acetic acid. The crude **1** was then purified by flash column chromatography. HPLC was checked to confirm the purity (elution with trifluoroacetic acid and a 0–100% acetonitrile linear gradient (0–40 min) at a flow rate of 1.0 mL/ min under 254 nm). Yield (5 mg, 23%) (ESI-TOF-MS (positive) m/z calcd for $C_{76}H_{97}N_{25}O_{15}^{2+}$ $[M+2H]^{2+}$799.87; found 799.89.

Synthesis of IPA(OBn), (4). Isophthalic acid (1.66 g, 10 mmol) was dissolved in a mixture of 15 mL methanol and 1 mL water, stirred at room temperature. To the stirred solution triethylamine (1.4 mL, 10.1 mmol) dissolved in methanol (10 mL) was added. The reaction mixture was stirred at room temperature for 12 h. The solvent was removed by vacuum pump and the residue was dissolved in 15 mL of DMF. Benzyl bromide (1.3 mL, 11 mmol) was added very slowly and the reaction mixture was stirred for another 2 h at 100 ^{o}C. The reaction was quenched by dropping temperature at 0 ^{o}C and by adding 5 % sodium bicarbonate (50 mL). It was then extracted with EtOAc (3x30 mL). The aqueous layer was carefully acidified with 10% HCL to pH 5-6 and further extracted with EtOAc (2x30 mL) to remove the diester. The aqueous part was further acidified to pH 3 and again extracted with EtOAc (3x30 mL) to achieve **4**. To the organic layer Na_2SO_4 was added and then concentrated under reduced pressure to yield white powder (1.18 g, 46%). TLC was checked to confirm the purity. ^{1}H NMR (600 MHz, [d6] DMSO): δ = 8.79 (t, 1H), 8.32 (d, 1H), 8.30 (d, 1H), 8.29 (d, 1H), 7.56 (t, 2H), 7.47 (d, 2H), 7.41 (m, 1H), 5.40 (s, 2H).

Synthesis of IPA-tail, (5). To a solution of **4** (300 mg, 1.17 mmol) dissolved in DMF (1 mL), PyBOP (912 mg, 1.75 mmol) was added and stirred at room temperature for 5 min. To the reaction mixture 3,3-diamine *N*-methyldipropylamine (0.282 mL, 1.75 mmol) was added and stirred at room temperature for about an hour. TLC was analysed to check the status of the reaction and confirmed the complete disappearance of starting material. The reaction mixture were dissolved in 5% HCL and extracted with EtOAc (15 mLx2). To the aqueous layer saturated $NaHCO_3$ was added very slowly at ice bath till pH levels to 12-14. Again it was extracted with EtOAc (15 mLx2) and then concentrated by rota vapour. The purity was checked using HPLC (elution with trifluoroacetic acid and a 0–100% acetonitrile linear gradient (0–20 min) at a flow rate of 1.0 mL/ min under 254 nm). Yield (358 mg, 80%) (ESI-TOF-MS (positive) m/z calcd for $C_{22}H_{29}N_3O_3$ $[M+H]^+$ 384.23; found 384.20. ^{1}H NMR (600 MHz, [d6] DMSO): δ = 8.66 (s, 1H), 8.46 (s, 1H), 8.10 (d, 2H),8.06 (d, 2H), 7.47 (s, 1H), 7.37 (m, 2H), 7.28 (m, 2H), 5.31 (s, 2H), 3.49 (br, 2H) 3.39 (s, 2H), 3.12 (br, 2H) 2.89 (s, 2H), 2.74 (s, 3H), 2.04 (br s, 2H), 1.97 (br s, 2H).

Boc-β-β-Im-Py-Py-Py-γ-Im-Py-Py-Py-COOH (6). After the solid phase synthesis the resin (60 mg) were added to 400 μL of 2 M NaOH and later 100 μL of 1,4-dioxane were added, stirred at 55 ^{o}C for 3 h. Filtered out the resin and the filtrate was concentrated by vacuum pump. To the crude yellow paste like material, Et_2O was added for precipitation with very little amount of H_2O. Washed 2-3 times by Et_2O and centrifugation gives a powder form of the compound **6** (20 mg). ESI-TOF-MS (positive) m/z calcd for $C_{61}H_{74}N_{21}O_{14}$ $[M+H]^+$ 1324.57; found 1324.98.

Boc-β-β-Im-Py-Py-Py-γ-Im-Py-Py-Py-L-IPA(OBn) (7). Conjugate ***6*** (17 mg) was dissolved in 0.5 mL of DMF. To it, PyBOP (3 equiv.) and DIEA (3 equiv.) were added, stirred for 10 min at room temperature. Followed by **5** (5 equiv.) were added to the reaction mixture and stirring was continued for 2h. After the completion of the reaction the solvent were removed by vacuum pump and excess Et_2O were added for precipitation. The crude product **7** washed several times with DCM and Et_2O to get powder form**.** Dried in desiccator and HPLC were checked to confirm the purity. ESI-TOF-MS (positive) m/z calcd for $C_{83}H_{102}N_{24}O_{16}$ $[M+2H]^+$ 845.395; found 845.5.

SAHA-β-β-Im-Py-Py-Py-γ-Im-Py-Py-Py-L-IPA, (2). Conjugate **7** (12 mg) was dissolved in methanol (3 mL). To it, Pd-C (5 mg) was added and then H_2 gas was passed into the reaction vessel using balloon, stirred at room temperature for 12 h. It was then filtered and concentrated using vacuum pump. In the next step, 10% DCM in TFA was used to cleave boc group. After the boc cleavage it was then coupled with HOOC-SAHA(Ome) using PyBOP and DIEA. At the final step the crude product having a methyl ester were dissolved in 150 μL of NMP and another 150 μL of aqueous 50% (v/v) NH_2OH, stirred at room temperature for 12 h. The reaction was quenched by adding acetic acid and purified by HPLC. Final purity was checked

using HPLC (elution with trifluoroacetic acid and a 0–100% acetonitrile linear gradient (0–40 min) at a flow rate of 1.0 mL/ min under 254 nm) (t_R= 19.4 min). Yield (2.7 mg, 13.5%). ESI-TOF-MS (positive) m/z calcd for $C_{86}H_{106}N_{26}O_{18}$ $[M+2H]^{2+}$ 895.41; found 895.38.

Ac-Im-Py-Py-Py-γ-Im-Py-Py-Py-Dp (3). After SPPS, which having a free amine group at the N-terminus end, was washed with 20% chloroacetic anhydride/DMF for 30 min for capping. Followed by detached from the resin using N, N-dimethylaminopropylamine (Dp), stirred at 45 °C for 3h to form 3. Final purification was done by flash column chromatography. Purity was checked by HPLC (elution with trifluoroacetic acid and a 0–100% acetonitrile linear gradient (0–40 min) at a flow rate of 1.0 mL/ min under 254 nm) (t_R= 15.6 min). Yield (2.7 mg, 14%).ESI-TOF-MS (positive) m/z calcd for $C_{57}H_{69}N_{21}O_{10}$ $[M+2H]^{2+}$ 604.80; found 604.79.

Cell Culture and Polyamide Treatment to MEF

MEF cells used were from its sixth passage, were trypsinized for 5 min at 37°C and then resuspended in fresh DMEM medium for making a concentration of 1.5×10^5 cell/mL in a 30 mm dish culture plate. After overnight incubation for attachment, the medium was replaced with fresh DMEM (2mL) containing polyamide conjugates in 100 nM concentration. In the CD study first **1** was dissolved in 1:1 mixture of 2x PBS and either 5 or 50 mM of CD followed by sonication for 10 min and then equilibrated for about an hour prior to the treatment in MEF. DMSO (0.1%) treated MEF was used for control experiment and ES cells used as a positive control. All the plates were incubated in a 5% CO_2 humidified atmosphere at 37°C. MEF treated with were harvested at 24h based on the previous studies.

Quantification of expression of marker genes in MEF: Total RNA was extracted from polyamide and DMSO treated MEF using an RNeasy Mini Kit (Qiagen) and cDNA was synthesized with ReverTra Ace qPCR RT kit (Toyobo, Japan), in accordance with the manufacturer`s protocol. SYBR green real-time RT-PCR amplifications were carried out in triplicate with the protocol and conditions mentioned in THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan) on an ABI 7300 Real-Time Detection System (Applied Biosystems, USA) and were analyzed using a 7300 System SDS Software v1.3.0 (Applied Biosystems, USA). Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After normalization with housekeeping gene GAPDH, using the comparative cycle threshold (CT) method, relative expression level of each gene was analyzed by considering the gene expression in DMSO treated cells as 100%. Primer pairs used for the endogenous genes such as Oct-3/4, Nanog, Sox2, Klf4, Dppa4, Cdh1, Rex1 and c-Myc were reported before.[10][19]

Details regarding the HPLC data, compound characterization by TOF mass and 1H NMR images are provided in the supplementary data.

Acknowledgements

We thank the Ministry of Education, Culture, Sports, Science and Technology in JAPAN (MEXT), administrated by the Japan Society for the Promotion of Science for the financial support. AS thank Seiwa International Scholarship Foundation for partial financial support in his doctoral program.

Keywords: SAHA Polyamide · Histone deacetylase· Cellular Uptake· Polyamide solubility · C-terminus modification

[1] P. Hou, Y. Li, X. Zhang, C. Liu, J. Guan, H. Li, T. Zhao, J. Ye, W. Yang, K. Liu, J. Ge, J. Xu, Q. Zhang, Y. Zhao, H. Deng, *Science* **2013**, *341*, 651-654.

[2] a) Y-L. Wu, G. N. Pandian, Y-P. Ding, W. Zhang, Y. Tanaka, H. Sugiyama, *Chem. Biol.* **2013**, *20*, 1311-1322. b) S. Masuda, J. Wu, T. Hishida, G. N. Pandian, H. Sugiyama, J. C. I. Belmonte, *J. Mol. Cell Biol.* **2013**, *5*, 354-355.

[3] A. Ohtsuki, M. T. Kimura, M. Minoshima, T. Suzuki, M. Ikeda, T. Bando, H. Nagase, K. Shinohara, H. Sugiyama, *Tetrahedron Lett.* **2009,** *50*, 7288–7292.

[4] U. Wahnert, O. Zimmer, G. Luck, O. Pitra, *Nucleic Acids Res.* **1975**, *2*, 391–404.

[5] M. L. Kopka, C. Yoon, D. Goodsell, P. Pjura, R. E. Dickerson, *Mol. Biol.* **1985**, *183*, 553–563.

[6] P. B. Dervan, *Bioorg. Med. Chem.,* **2001**, *9,* 2215-2235.

[7] L. Han, G. N. Pandian, S. Junetha, S. Sato, C. Anandhkumar, J. Taniguchi, A. Saha, T. Bando, H. Nagase, H. Sugiyama, *Angew. Chem. Int. Ed.* **2013**, *52*, 13410-13413.

[8] G. N. Pandian, J. Taniguchi, S. Junetha, S. Sato, L. Han, A. Saha, C. Anandhkumar, T. Bando, H. Nagase, V. Thangavel, R. D. Taylor, H. Sugiyama, *Sci. Rep.* **2014**, *4*, 3843.

[9] G. N. Pandian, et al. *ChemBioChem,* **2011**, *12*, 2822-2828.

[10] G. N. Pandian, Y. Nakano, S. Sato, H. Morinaga, T. Bando, H. Nagase, H. Sugiyama, *Sci. Rep.* **2012**, *2*: 544.

[11] C. S. Jacobs, and P. B. Dervan, *J. Med. Chem.* **2009,** *52*, 7380–7388.

[12] A. E. Hargrove, J. A. Raskatov, J. L. Meier, D.C. Montgomery, and P. B. Dervan, *J. Med. Chem.* **2012**, *55*, 5425−5432.

[13] J. M. Belitsky, S. J. Leslie, P. S. Arora, T. A. Beerman, P. B. Dervan, *Bioorg. Med. Chem.* **2002**, *10*, 3313–3318.

[14] N. G. Nickols, C. S. Jacobs, M. E. Farkas, P. B. Dervan, *Nucleic Acids Res.* **2007**, *35*, 363–370.

[15] R.M. Adlington, J.E Baldwin, G.W. Becker, B. Chen, L. Cheng, S.L. Cooper, R.B. Hermann, T.J. Howe, W. McCoull, A.M. McNulty, B.L. Neubauer, G.J. Pritchard. *J. Med. Chem.* **2001**, *44*, 1491-1508

[16] D. M. Chenoweth, D. A. Harki, and P. B. Dervan. *J. Am. Chem. Soc.* **2009**, *131*, 7175–7181.

[17] S. Nishijima, K. Shinohara, T. Bando, M. Minoshima, G. Kashiwazaki, H. Sugiyama, *Bioorg. Med. Chem.* **2010**, *18*, 978–983.

[18] J. L. Meier, D. C. Montgomery, and P. B. Dervan, *Nucleic Acids Res.,* **2012**, *40*, 2345–2356

[19] K. Takahashi, S. Yamanaka, *Cell* **2006**, *126,* 663-76.

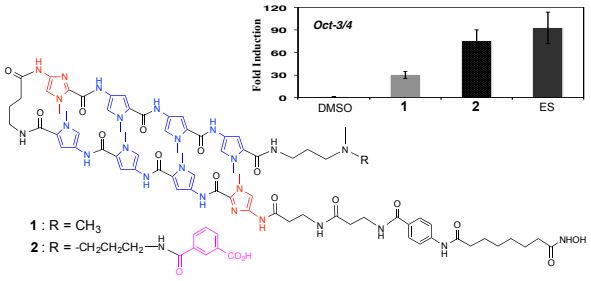
Received: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPERS

Targeted transcriptional activator is crucial for cellular reprogramming. We identified compound **1** can induce Oct3/4 pathway genes related embryonic stem cell pluripotency. Here we report for the first time that chemical modification of **1** at the C-terminus can significantly enhance its biological efficacy similar to the ES cell.



Abhijit Saha,[a] Ganesh N. Pandian,[b] Shinsuke Sato,[b] Junichi Taniguchi, [a] Yusuke Kawamoto,[a] Kaori Hashiya, [a] Toshikazu Bando,[a] and Hiroshi Sugiyama[a],[b]*

Page No. – Page No.

Chemically Modified Synthetic Small Molecule Boosts its Biological Efficacy against Pluripotency Genes in Mouse Fibroblast