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Original article

Histone deacetylase inhibitor prodrugs in nanoparticle vector enhanced gene expression in human cancer cells

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ABSTRACT

We developed histone deacetylase inhibitor (HDACI) prodrugs to enhance the expression of the external genes transfected into human cells with cationic nanoparticles (NPs). We synthesized five kinds of lipid-linked HDACI prodrugs in which *n*-dodecanoic acid or cholesterol is linked with a potent HDACI, K-182, by an ester bond or a disulfide carbonate linker. The prodrugs were able to admix as a component of NPs, although the intact K-182 was not incorporated into NPs. Namely, NPs composed of cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine and Tween 80 with the 10 mol% K-182 prodrug were prepared as a DNA vector to transfect plasmid DNAs into human prostate cancer cells, PC-3, or human breast cancer cells, Sk-Br-3. The NPs containing K-182 prodrugs with *n*-dodecanoic acid exhibited two to four times higher the gene expression than the original NPs. The enhancement of the gene expression will be due to the hyperacetylation of core histones caused by intact K-182 degraded from the prodrug in the vector incorporated into the cells.

1. Introduction

Development of an efficient and safe method for the delivery of exogenous DNAs into mammalian cells is critical both for basic biosciences and clinical applications, including iPS cell techniques and gene therapy. Viral vectors have been used for this purpose because of their high transfection efficiency, but disadvantages such as pathogenicity, immunogenicity, mutational potential and low capacity of gene carrying have led to the search of a non-viral delivery system [1–4]. Among the non-viral systems, lipofection using cationic cholesterol derivatives is considered to be safer since it is less immunogenic and has low toxicity [5,6]. We reported that cationic nanoparticles (NPs) composed of cholesteryl-3β-carboxyamidoethylene-*N*-hydroxyethylamine (OH-Chol, Fig. 1) [5] and Tween 80 could deliver plasmid DNA into cells with high transfectional efficiency [7]. Cationic NPs bind electrostatically to negatively charged DNAs to form complexes called nanoplexes, which can enter the cells via endocytosis [8,9] followed by the escape from the endosomes into the cytoplasm and transfer into the nucleus [10].

In order to deliver DNAs efficiently to the nucleus and achieve high levels of expression, there are several obstacles that must be overcome even though they have successfully intruded into the cytoplasm [11,12]. We assumed that for the further development of more efficient NP vector, improvements have to be made to the processes of intracellular DNA delivery and intranucleus DNA transcription. Recently, it was reported that inhibition of histone deacetylase 6 (HDAC6) increased transfection efficiency due to the improved gene transfer in cytoplasm caused by hyperacetylation of microtubules [13]. It is also known that many kinds of HDAC inhibitors (HDACIs) augment the expression of genes because of the hyperacetylation of subnuclear core histone proteins. We therefore attempted to associate the function of NP with HDACI for the development of highly efficient vectors.

There are three different chemical types in major HDACIs, i.e. the fatty acid-type, benzamide-type and hydroxamate-type. Fatty acid-type HDACIs, such as butylate, or valproate [14], may have affinity to NPs, however, the HDACI activity of them is three orders of magnitude lower than the other types. Since, the benzamide-type HDACIs such as MS275 [15] and MGCD0103 [16] are selective inhibitor of class I HDACs, the improvement of DNA transport in the cytoplasm cannot be expected because the class II HDAC, namely HDAC6, may not be inhibited by them [17]. On the other hand, the hydroxamate-type HDACIs such as trichostatin A [18], suberoylanilide hydroxamic acid (SAHA, vorinostat) [19] and our K-series compounds [20–22] have potent inhibitory effects against broad HDAC isoenzymes, including cytoplasmic HDAC6 and nuclear

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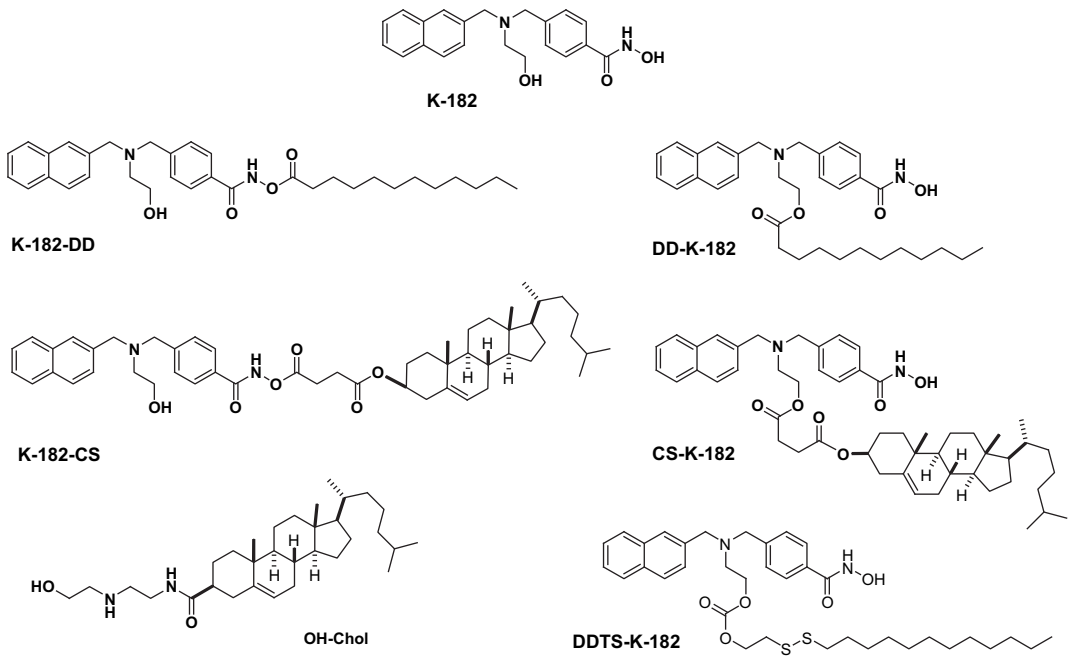


Fig. 1. Chemical structures of K-182, K-182 prodrugs and OH-Chol.

HDAC1. We therefore assessed the compatibility of the hydroxamate-type HDACIs as a component of NP formulation. However, unsatisfactory results were obtained from admixed formulation of the HDACIs and NP, namely a considerable segregation of HDACIs occurred. Hence, we attempted to synthesize HDACI prodrugs compatible to NP. Taking into consideration the diverse derivatization, we selected one of the K-series compounds, K-182, (Fig. 1) [20] which has two OH groups to tether other groups with a biodegradable ester bond.

In this study, five kinds of K-182 prodrugs, DD–K-182, DDTS–K-182, CS–K-182, K-182–DD and K-182–CS, (Fig. 1) were synthesized to evaluate their compatibility to NP formulation and their ability to enhance the expression of the transfected genes.

2. Chemistry

We tried to form NPs containing hydroxamate-type HDACIs, but we decided against this because of the segregation of the compounds during preparation. To overcome this problem, we attempted to form HDACI prodrugs which have affinity to OH-Chol, the major component of NP. Regarding the activity and feasibility of derivatization, we selected K-182 as a substrate of the prodrugs.

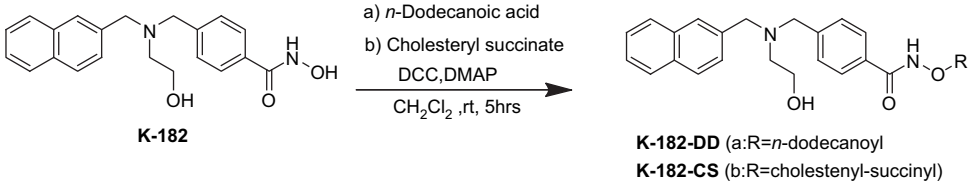
K-182 has two hydroxyl (OH) groups that can link with other functional groups via ester bonds. We designed K-182 prodrugs in which K-182 is tethered to an aliphatic fatty acid or cholesteryl group via biodegradable bonds or linkers. When non-protected K-182 was treated with equimolar *n*-dodecanoic acid (lauric acid) with the coupling regent, DCC–DMAP, *n*-dodecanoic acid was selectively coupled to an OH group in hydroxamic acid to form mono-dodecanoate, K-182–DD, due to the higher acidity of OH in hydroxamic acid than in alcoholic OH in the hydroxyethyl group (Scheme 1). On the other hand, coupling of *n*-dodecanoic acid to a hydroxyethyl group was performed using a substrate in which OH in a hydroxamic acid was protected with tetrahydropyranyl ether (THP). After the coupling reaction, THP was deprotected with acetic acid in THF–H_2O at 60 °C for 10 h to give a monoester DD–K-182 with an excellent yield (Scheme 2). Two kinds of cholesteryl succinate monoesters of K-182, K-182–CS and CS–K-182, were synthesized by coupling cholesteryl succinate in the same manner as the coupling of *n*-dodecanoic acid (Scheme 2 and 3).

We also attempted to connect an aliphatic thiol to hydroxyethyl moiety in K-182 via a disulfide carbonate linker which is designed to release unmodified K-182 in the reductive cytosolic condition as shown in the route in Scheme 3 [23]. The mixed disulfide, (2-pyridinyldithio)ethyl carbonate **3**, for the subsequent coupling with aliphatic thiol was synthesized with the reaction of **1** and triphosgene, followed by condensation of 2-(2-pyridinyldithio) ethanol [24] to the acid chloride intermediate. Coupling of dodecanethiol to **3** followed by deprotection of THP afforded the target molecule DDTS–K-182 (Scheme 4). Accordingly, five kinds of K-182 prodrugs were prepared (Fig. 1).

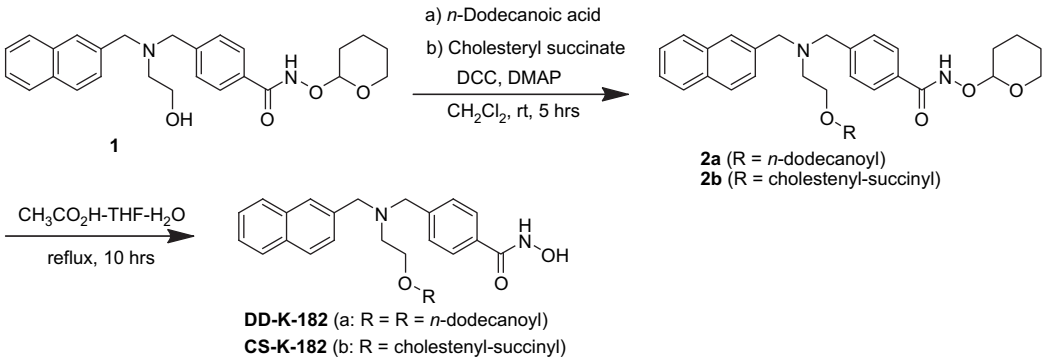
3. Results and discussion

3.1. Optimization of concentration of K-182 prodrugs in NP and of concentration of the NPs in the medium affected to PC-3-Gluc cells

In order to optimize the formulation, NPs containing K-182–DD with different proportions were prepared using the modified ethanol injection method as previously described [7] (Table 1). The



Scheme 1.



Scheme 2.

formulations were based on the original NP, yet 5, 10 or 20 mol% of OH-Chol in the original was displaced by K-182–DD (NP-5K, NP-10K and NP-20K, respectively). Subsequently, the resulting water suspension of the NPs (1 mg/mL) was incubated with plasmid DNAs for 10 min at room temperature to form nanoplexes (DNA/NP complexes). According to our previous study, the ratio between DNA and total NP is fixed so as to form nanoplexes at a charge ratio (+/−) of 3/1 of cationic NP to plasmid DNA pGL3-basic encoding luciferase gene without promoter, corresponding to a concentration ratio of NPs (μM) to DNA (μg/mL) of 10 throughout this experiment [25]. The nanoplexes were diluted with a cell growth medium to prepare the transfection medium containing 5, 10, 20 and 40 μM NPs with twofold serial dilution of the initial 40 μM NPs. In this optimization, PC-3 cells stably expressing *Gaussia* luciferase (PC-3-Gluc cells) were used to evaluate the effect of K-182–DD in each NP on the gene expression, excluding the factor of transfection. The cells were incubated with each medium for 24 or 48 h and the increased expression of the *Gluc* gene was evaluated using luciferase assay (Fig. 2).

Increased expression of the *Gluc* gene was observed significantly in the cells incubated with the medium containing 5–20 μM NP-10K and NP-20K both for 24 h and 48 h incubation times. (Fig. 2A and B) NP formulations of 40 μM, however, did not show the augmentation effect of K-182–DD, probably because of the higher cytotoxicity caused by higher concentration of OH-Chol and K-182–DD. Although 20 μM NP-20K seems optimum for the gene expression, the cytotoxicity in that condition was more significant (cell viability: 19.6 ± 1.4% of total cells as mean ± S.D.) compare to that of control NP or NP-10K (cell viability: 49.4 ± 2.2% and 34.3 ± 2.5%, respectively) at the same concentration ($P < 0.01$). We therefore chose the transfection media containing 20 μM NPs and we prepared NPs with 10 mol% of DD–K-182, CS–K-182, K-182–CS, and DDTS–K-182, as well as K-182–DD in further experiments. Their average particle sizes and ζ-potentials are in the range of 137.9–176.7 nm (polydispersity index: 0.1–0.2) and 64.0–63.0 mV, respectively and those of control NPs are 165.3 nm (polydispersity index: 0.02) and 48.8 mV, respectively. Replacement of 10 mol% of OH-Chol to K-182 prodrugs increased the surface potential of NPs, probably because of a tertiary amine, that can be positively charged, in K-182 moiety exposed on the surface of NPs.

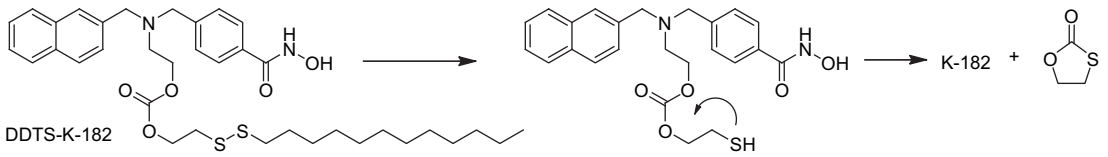
3.2. Effect of K-182 prodrugs contained in NPs on expression of externally transfected genes

Nanoplexes were formed by the incubation of NPs with plasmid pCMV-Gluc control encoding secretable *Gaussia* luciferase (Gluc) under the control of the CMV promoter. According to the optimization mentioned above, nanoplexes were diluted to form the transfection medium of 20 μM NPs, containing 2 μM K-182 prodrugs at a charge ratio (+/−) of 3/1 of cationic NP to plasmid DNA, and were transfected to PC-3 cells. As shown in Fig. 3, NPs containing 10 mol% DD–K-182, K-182–DD and DDTS–K-182 (NP–DD–K-182, NP–K-182–DD and NP–DDTS–K-182) augmented the luciferase expression 2–3.5 times more than control NP. However, NPs containing 10 mol% cholesteryl ester, CS–K-182 and K-182–CS (NP–CS–K-182 and NP–K-182–CS) did not increase the expression. It is interesting that the addition of 2 μM K-182 to the medium containing control NP did not potentiate the expression of transfected genes. Similar results were obtained when Sk-Br-3 cells were used as a host for the transfection with NP–DD–K-182, NP–K-182–DD and NP–DDTS–K-182, i.e. they enhanced the expression about three times more than control NP. The addition of external K-182 did not increase the expression, in these cells. The simultaneous incorporation of DNA and K-182 prodrugs into the cells with NPs will be critical for the activation of gene expression.

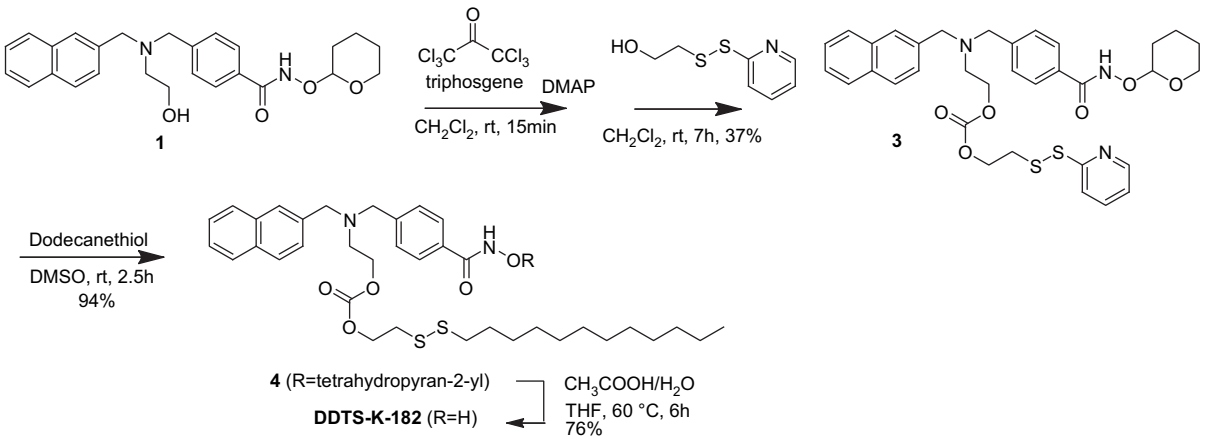
3.3. Effect of NPs composed of K-182 prodrugs on acetylation of histone H3 in human prostate tumor PC-3 cells

Histone H3 is one of the core histone proteins in the chromatin of eukaryotic cells. Hyperacetylation of lysine residues, i.e. lysine 9, in the N-terminal tails of histone H3 loosens the histone–DNA binding and activates gene transcription. On the other hand, deacetylation of acetylated lysine residues leads to tight histone–DNA binding, which restricts the access of transcriptional factors. Histone acetyltransferases (HATs) and HDACs play a crucial role in this reversible acetylation and deacetylation of histones regulating gene expression. Inhibition of HDACs, therefore, induces histone hyperacetylation and activates gene transcription [26–28].

Prior to examine the acetylation of cellular histone H3, we tested the effect of intact K-182 prodrugs, K-182–DD, DD–K-182 and



Scheme 3. Disulfide mediated release of K-182 from the carbonate linker.



Scheme 4.

DDTS–K-182 on HDAC activity. As shown in Table 2, K-182 prodrugs are inactive ($IC_{50} > 10\ \mu M$), although K-182 exhibited IC_{50} of 0.90 μM. The activity of HDACI was recovered when DDTS–K-182 was treated with dithiothreitol, indicating the release of K-182 by degradation of disulfide carbonate linker with reductive conditions.

We tested the effect of nanoplexes containing K-182 prodrugs on the acetylation of core histone H3 protein in human prostate tumor cells, PC-3, using western blot analysis detecting the acetylation on Lys-9 side chains. Incubation of the cells with nanoplexes formed with NP–DDTS–K-182, NP–DD–K-182 or NP–K-182–DD resulted in increased levels of acetylated histone H3 protein in the cells to a similar extent as those increased with 2 μM K-182 or another type of HDACI K-32 [22] in the medium with or without the presence of NP–pGL3-basic nanoplexes (Fig. 4). This observation, together with the result that intact prodrugs are inactive in HDAC inhibition, suggesting that free K-182 is efficiently released from the complexes of prodrugs in the cells and inhibits HDAC in the nucleus resulting in the hyperacetylation of histone H3.

It remains controversial that addition of K-182 externally to the medium did not increase the expression of transfected genes in PC-3 cells as shown in Fig. 3, even though the hyperacetylation of histone H3 in the cells was observed in the similar condition (Fig. 4) and the fact that addition of K-182 to PC-3-Gluc cells, on the other hand, increased the expression of *Gluc* gene stably expressed in the cells (Fig. 5). The simultaneous delivery of K-182 prodrugs and genes into the cells with the formulation of nanoplexes is probably important to enhance the outer gene expression.

4. Conclusion

In conclusion, cationic NPs composed of 10 mol% K-182 prodrugs, K-182–DD, DD–K-182 and DDTS–K-182, with 85 mol% OH-Chol and 5 mol% Tween 80 were prepared as a DNA vector to transfect plasmid DNA into human prostate cancer cells, PC-3, or human breast cancer cells, Sk-Br-3. These NPs exhibited expression of the genes two to four times more efficiently than those with the original NP. The enhancement of the gene expression will be due to the hyperacetylation of core histones caused by K-182 derived from K-182 prodrugs in the NP formulation.

Table 1
Formulae of NPs containing K-182–DD.

NP formulation[a]	Components (mol%)		
	OH-Chol	Tween 80	K-182–DD
Control NP	95	5	0
NP-5K	90	5	5
NP-10K	85	5	10
NP-20K	75	5	20

[a] NPs were prepared with a cationic cholesterol (OH-Chol), Tween 80 and K-182–DD using modified ethanol injection method [7] as 1 mg/mL suspension in water.

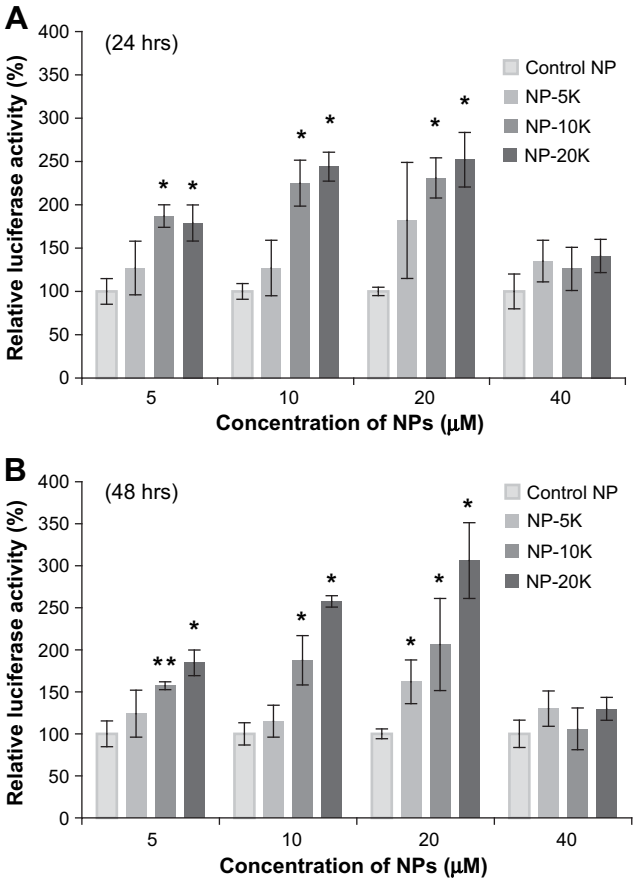


Fig. 2. Effect of concentration of K-182–DD in NPs on increased expression of *Gluc* gene stably expressed in PC-3-Gluc cells at a charge ratio (+/−) of 3/1 of cationic NP to plasmid DNA (corresponding to a concentration ratio of NPs (μM) to DNA (μg/mL) of 10). PC-3-Gluc cells were incubated for 24 h (A) or 48 h (B) with cell growth media containing nanoplexes formed with 5, 10 and 20 mol% K-182–DD in NPs; NP-5K, NP-10K or NP-20K. Each column represents the mean ± S.D. ($n = 3$). *$P < 0.01$, significantly different from control.

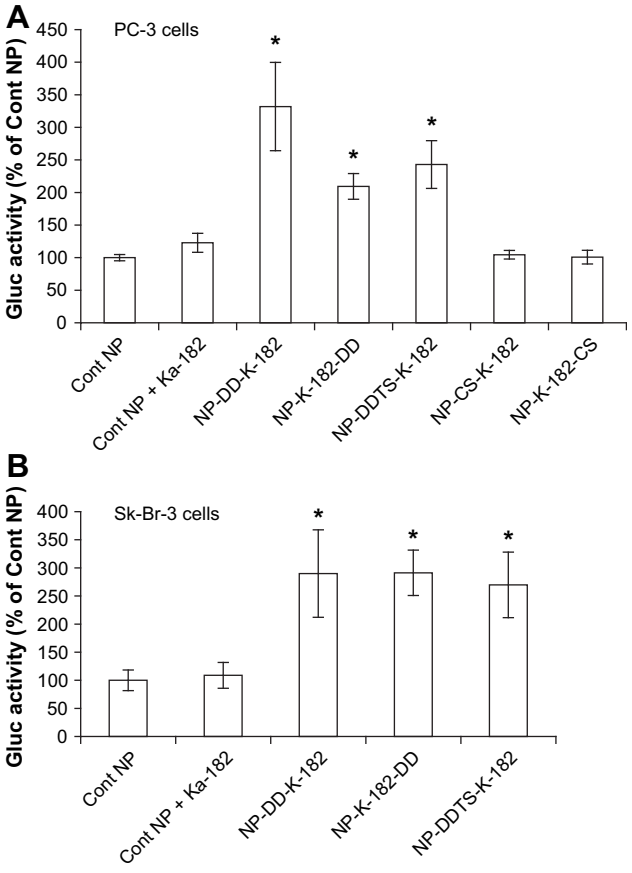


Fig. 3. Effects of 10 mol% K-182 prodrugs contained in NPs on the expression of genes transfected to PC-3 cells (A) and Sk-Br-3 cells (B). PC-3 cells or Sk-Br-3 cells were incubated for 24 h in the transfection medium containing nanoplexes formed with 20 μM NPs and 2 μg/mL plasmid DNA, pCMV-Gluc. For the control, the media containing or not containing 2 μM K-182 were used for transfection with the control NP. Each column represents the mean ± S.D. ($n = 3$). *$P < 0.01$, significantly different from control.

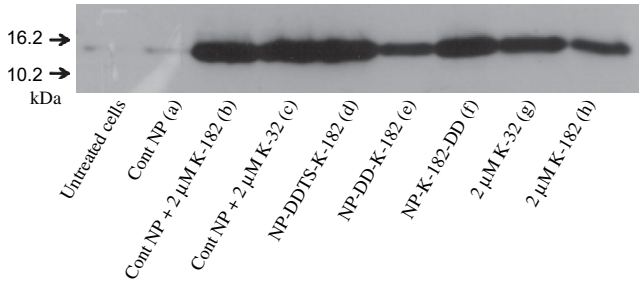


Fig. 4. Acetylation of core histone H3 protein in PC-3 cells induced by HDACIs (K-32 and K-182) and NPs composed of K-182 prodrugs. The cells were treated with a medium containing (a–f) or not containing (g, h) NP–pGL3-basic nanoplexes in the presence (b–h) or absence (a) of the HDACIs. The medium containing the nanoplexes (a–f) was formed with pGL3-basic plasmid DNA (2 μg/mL) and 20 μM NP (a–c), NP–DDTS–K-182 (d), NP–DD–K-182 (e) or NP–K-182–DD (f). After 24 h incubation of the medium, cellular proteins were isolated from the cells and were resolved on a 12% SDS–PAGE (each 3 μg protein in a well), followed by Western blot analysis for acetylated histone H3 protein.

5. Experimental

5.1. Synthesis of K-182 prodrugs

Melting points were determined on a Yanagimoto MP-32 micromelting point apparatus and are uncorrected. IR spectra were recorded on Shimadzu FTIR-8400 infrared spectrophotometer. FAB-MS spectra were measured on a JEOL JMS-HX 100 instrument. ^{1}H and ^{13}C NMR spectra are recorded on JEOL EX-400 (399.7 MHz for ^{1}H NMR and 100.4 MHz for ^{13}C NMR) instruments using tetramethylsilane as an internal standard. Analytical and preparative TLC were performed using Silica gel 60 F254 (Merck, 0.25 and 0.5 mm, respectively) glass plates. Column chromatography was performed using Silica Gel 60 (70–230 mesh ASTM).

5.1.1. N-(Dodecanoyloxy)-4-{[(2-hydroxyethyl)(2-naphthylmethyl)-amino]methyl}benzamide (K-182–DD)

To a solution of K-182 (33.2 mg, 94.6 μmol) in CH_2Cl_2 (4.0 mL) were added *n*-dodecanoic acid (19.0 mg, 94.6 μmol), DCC (19.5 mg, 94.6 μmol) and DMAP (11.6 mg, 94.6 μmol). After being stirred for 5 h at rt, the precipitate was filtered off and the filtrate was concentrated in vacuo. Purification of the resulting residue by silica gel column chromatography (eluent: ethylacetate/hexane = 1/1) gave K-182–DD (12.2 mg, 23.0 μmol, 24.3% yield) as a colorless oil. IR ($CHCl_3$) γ/cm^{-1}: 3341, 3010, 2927, 2854, 1780, 1697, 14,111, 1141, 773; ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 3H, $J = 7.2$ Hz, $\underline{CH_3}CH_2$), 1.0–1.4 (m, 16H, $-(CH_2)_8-$), 1.60–1.75 (m, 2H, $COCH_2\underline{CH_2}$), 2.55 (t, 2H, $J = 7.6$ Hz, $COCH_2$), 2.71 (t, 2H, $J = 5.2$ Hz, $NC\underline{H_2}CH_2$), 3.62 (t, 2H, $J = 5.2$ Hz, $HOC\underline{H_2}CH_2$), 3.70 (s, 2H, NCH_2–naph), 3.78 (s, 2H, NCH_2Ph), 7.41–7.52 (m, 5H, Ar–H), 7.71–7.84 (m, 6H, Ar–H); ^{13}C NMR ($CDCl_3$) δ: 14.10, 22.66, 24.70, 24.90, 25.59, 28.99, 29.14, 29.31, 29.37, 29.56,

Table 2
Inhibitory effect of HDACIs and K-182 prodrugs on HDAC.

Compounds	IC_{50} (μM)[a,b]
TSA[c]	0.012
K-182	0.90
K-182–DD	>10
DD–K-182	>10
DDTS–K-182	>10
DDTS–K-182 + DTT[d]	2.7

[a] Inhibition of crude HDACs from nuclear extract of HeLa cells provided in CycLex HDAC Assay Kit.
[b] Assays were performed in duplicate.
[c] Trichostatin A (TSA) as positive control of HDACI.
[d] DDTS–K-182 was preincubated with equimolar dithiothreitol (DTT) in DMSO for 2 h before addition to the assay buffer.

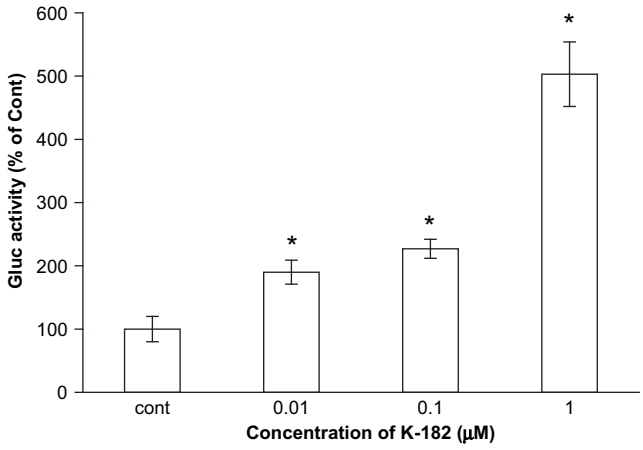


Fig. 5. Effect of K-182 on the expression of genes stably expressed in PC-3-Gluc cells. PC-3-Gluc cells were incubated for 24 h with cell growth media with or without K-182. Each column represents the mean ± S.D. ($n = 3$). *$P < 0.01$, significantly different from control.

31.78, 31.89, 33.90, 55.11, 58.03, 58.61, 58.77, 125.85, 126.19, 126.86, 127.63, 127.67, 127.71, 127.80, 128.37, 129.29, 129.78, 132.84, 133.29, 135.86, 144.22, 172.20; HR-FAB-MS *m*/*z*: 533.3370 (calcd for $C_{33}H_{45}N_2O_4$, 533. 3379).

5.1.2. Cholest-5-en-3-yl 4-{[(4-{[(2-hydroxyethyl)(2-naphthylmethyl)amino]methyl}benzoyl) amino]oxy}-4-oxobutanoate (K-182–CS)

To a solution of K-182 (21.2 mg, 60.5 μmol) in CH_2Cl_2 (2.0 mL) were added cholesteryl succinate (29.5 mg, 60.5 μmol), DCC (12.5 mg, 60.5 μmol) and DMAP (7.4 mg, 60.5 μmol). After being stirred for 6 h at rt, the precipitate was filtered off and the filtrate was concentrated in vacuo. Purification of the resulting residue by preparative SiO_2 TLC (eluent: ethylacetate/hexane = 4/5) gave K-182–CS (10.5 mg, 12.8 μmol, 21.2% yield) as a colorless oil. IR ($CHCl_3$) γ/cm^{-1}: 3320, 2949, 2868, 1759, 1724, 1466, 1364, 1105, 667; ^{1}H NMR ($CDCl_3$) δ: 0.67 (s, 3H, 18′-CH_3), 0.85 (d, 3H, J = 1.6 Hz, 26′ or 27′-CH_3), 0.87 (d, 3H, J = 1.6 Hz, 26′ or 27′-CH_3), 0.90–2.05 (m, 26H), 0.91 (d, 3H, J = 6.4 Hz, 21′-CH_3), 1.01 (s, 3H, 19′-CH_3), 2.32 (d, 2H, J = 7.6 Hz, 4′-CH_2), 2.71 (m, 4H, $COCH_2CH_2CO$), 2.87 (t, 2H, J = 6.8 Hz, NCH_2CH_2), 3.62 (t, 2H, J = 5.6 Hz, $HOCH_2CH_2$), 3.70 (s, 2H, NCH_2–naph), 3.78 (s, 2H, NCH_2–Ph), 4.63 (m, 1H, 3′-CH), 5.36 (m, 1H, 6′-CH=C), 7.41–7.48 (m, 5H, Ar–H), 7.70–7.83 (m, 6H, Ar–H); ^{13}C NMR ($CDCl_3$) δ: 11.85, 18.70, 19.28, 21.02, 22.54, 22.80, 23.83, 24.27, 27.05, 27.69, 28.00, 28.21, 29.14, 31.84, 31.89, 35.78, 36.18, 36.58, 36.94, 38.00, 39.52, 39.73, 42.32, 50.01, 55.17, 56.16, 56.69, 58.07, 58.65, 58.82, 74.79, 122.77, 125.84, 126.19, 126.87, 127.64, 127.68, 127.71, 127.79, 128.36, 129.29, 129.61, 132.86, 133.30, 135.87, 139.49, 144.29, 171.06; HR-FAB-MS *m*/*z*: 819.5320 (calcd for $C_{52}H_{71}N_2O_6$, 819.5234).

*5.1.3. 4-{[(2-Hydroxyethyl)(2-naphthylmethyl)amino]methyl}-N-(tetrahydro-2H-pyran-2-yloxy) benzamide (**1**)*

To a mixture of 4-{[(2-naphthylmethyl)amino]methyl}-*N*-(tetrahydro-2*H*-pyran-2-yloxy) benzamide [20] (1.0 g, 0.94 mmol) and Et_3N 0.71 mL (5.12 mmol) in CH_3CN (20 mL) was added 2-bromoethanol (0.36 mL,5.12 mmol). After stirring for 6 h at 60 °C, the mixture was evaporated to remove the solvent. Purification of the resulting residue by SiO_2 column chromatography (eluent: ethylacetate/hexane = 3/2) gave **1** as colorless amorphous (650 mg, 58.5% yield). IR (KBr) γ/cm^{-1}: 3221, 2936, 1719, 1653, 1275, 1204, 1051, 905; ^{1}H NMR ($CDCl_3$) δ: 1.62–1.86 (6H, m, $CH_2 \times 3$ of THP), 2.71 (2H, t, J = 5.6 Hz, N–CH_2–CH_2), 3.62 (2H, t, J = 5.6 Hz, CH_2–CH_2–OH), 3.64–3.68 (1H, m, –CH_a–O of THP), 3.69 (2H, s, N–CH_2–Ar), 3.78 (2H, s, naph–CH_2–N), 4.00 (1H, ddd, J = 11.2, 9.2, 2.8 Hz, –CH_b–O of THP) 5.07 (1H, t, J = 2.8 Hz, O–CH–O of THP), 7.38–7.84 (11H, m, Ar–H), 8.81 (1H, s, CO–NH–O); ^{13}C NMR ($CDCl_3$) δ: 18.61, 24.99, 28.03, 54.98, 57.97, 58.54, 58.71, 62.63, 102.65, 125.79, 126.14, 126.83, 127.37, 127.59, 127.67, 127.73, 128.29, 129.09, 130.97, 132.79, 133. 24, 135.91, 143.33; HR-FAB-MS *m*/*z*: 435.2289 (calcd for $C_{26}H_{31}N_2O_4$, 435.2284).

*5.1.4. 2-[(2-Naphthylmethyl)(4-{[(tetrahydro-2H-pyran-2-yloxy)amino]carbonyl}benzyl)amino] ethyl laurate (**2a**)*

To a solution of **1** (26.2 mg, 60.3 μmol) in CH_2Cl_2 (2.0 mL) were added *n*-dodecanoic acid (12.1 mg, 60.3 μmol), *N*,*N*-dicyclohexylcarbodiamide (DCC) (12.4 mg, 60.3 μmol) and *N*,*N*-dimethyl-4-aminopyridine (DMAP) (7.37 mg, 60.3 μmol). After being stirred for 5 h at rt, the precipitate was filtered off and the filtrate was concentrated in vacuo. Purification of the resulting residue by silica gel column chromatography (eluent: ethylacetate/hexane = 1/4) gave **2a** (22.4 mg, 36.3 μmol, 60.2% yield) as a colorless oil. IR ($CHCl_3$) γ/cm^{-1}: 3010, 2927, 1728, 1683, 1456, 1112, 777, 669; ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, J = 7.2 Hz, –CH_2CH_3), 1.25–1.28 (m, 16H, –$(CH_2)_8$–), 1.43–1.58 (m, 6H), 1.84–1.92 (m, 2H, $COCH_2CH_2$), 2.28 (t, 2H, J = 7.6 Hz, $COCH_2$), 2.77 (t, 2H, J = 5.6 Hz, NCH_2CH_2), 3.64–3.68 (1H, m, –CH_a–O of THP), 3.72 (s, 2H, NCH_2Ph), 3.78 (s, 2H, NCH_2), 3.95–4.10 (m, 1H, –CH_b–O of THP), 4.20 (t, 2H, J = 6.0 Hz, OCH_2CH_2N), 5.08 (m, 1H, OCHO), 7.43–7.52 (m, 5H, Ar–H), 7.70–7.83 (m, 6H, Ar–H); ^{13}C NMR ($CDCl_3$) δ: 14.09, 18.66, 22.66, 24.91, 25.02, 25.60, 28.06, 29.18 29.28, 29.31, 29.46, 29.59, 31.86, 33.91, 34.34, 51.91, 58.31, 58.89, 62.05, 62.69, 102.71, 125.62, 126.00, 126.92, 127.20, 127.30, 127.61, 127.65, 128.01, 128.88, 130.75, 132.80, 133.27, 136.56, 144.06, 173.74; HR-FAB-MS *m*/*z*: 617.3960 (calcd for $C_{38}H_{53}N_2O_5$, 617.3954).

*5.1.5. 1-Cholest-5-en-3-yl 4-{2-[(2-naphthylmethyl)(4-{[(tetrahydro-2H-pyran-2-yloxy) amino]carbonyl}benzyl)amino]ethyl} succinate (**2b**)*

To a solution of **1** (48.6 mg, 112 μmol) in CH_2Cl_2 (2.0 mL) were added cholesteryl succinate (54.4 mg, 112 μmol), DCC (23.0 mg, 111.6 μmol) and DMAP (13.6 mg, 111.6 μmol). After being stirred for 4 h at rt, the precipitate was filtered off and the filtrate was concentrated in vacuo. Purification of the resulting residue by preparative SiO_2 TLC (eluent: ethylacetate/hexane = 1/2) gave **2b** (66.3 mg, 73.4 μmol, 65.8% yield) as a colorless oil. IR ($CHCl_3$) γ/cm^{-1}: 3008, 2947, 2854, 1728, 1683, 1456, 1363, 1112, 761; ^{1}H NMR ($CDCl_3$) δ: 0.67 (s, 3H, 18′-CH_3), 0.85 (d, 3H, J = 1.6 Hz, 26′ or 27′-CH_3), 0.87 (d, 3H, J = 1.6 Hz, 26′ or 27′-CH_3), 0.9–2.1 (m, 32H), 0.91 (d, 3H, J = 6.4 Hz, 21′-CH_3), 1.01 (s, 3H, 19′-CH_3), 2.30 (d, 2H, J = 8.0 Hz, 4′-CH_2), 2.57 (m, 4H, $COCH_2CH_2CO$), 2.76 (t, 2H, J = 5.6 Hz, NCH_2CH_2), 3.63–3.67 (1H, m, –CH_a–O of THP), 3.69 (s, 2H, NCH_2Ph), 3.78 (s, 2H, NCH_2), 3.95–4.10 (m, 1H, –CH_b–O of THP), 4.20 (t, 2H, J = 5.6 Hz, OCH_2CH_2N), 4.62 (m, 1H, 3′-CH), 5.08 (m, 1H, OCHO of THP), 5.35 (s, 1H, 6′-CH=C), 7.43–7.53 (m, 5H, Ar–H), 7.70–7.82 (m, 6H, Ar–H); ^{13}C NMR ($CDCl_3$) δ: 11.83, 18.63, 18.70, 19.27, 21.00, 22.54, 22.80, 23.81, 24.26, 24.91, 25.04, 25.60, 27.71, 27.99, 28.07, 28.21, 29.41, 31.83, 31.88, 33.91, 35.76, 36.17, 36.55, 36.92, 38.04, 39.50, 39.71, 42.29, 49.99, 51.96, 56.12, 56.67, 58.31, 59.01, 62.44, 62.61, 74.43, 102.63, 122.71, 125.63, 126.02, 126.94, 127.24, 127.34, 127.62, 127.66, 128.06, 128.87, 130.82, 132.81, 133.27, 136.52, 144.01, 171.74, 172.17; HR-FAB-MS *m*/*z*: 903.5882 (calcd for $C_{57}H_{79}N_2O_7$: 903.5887).

5.1.6. 2-[{4-[(Hydroxyamino)carbonyl]benzyl}(2-naphthylmethyl)amino]ethyl laurate (DD–K-182)

To a solution of **2a** (16.5 mg, 26.8 μmol) in THF (1.0 mL) were added acetic acid (2.0 mL) and H_2O (0.5 mL). After refluxing for 10 h, the solution was concentrated in vacuo. Purification of the residue by preparative SiO_2 TLC (eluent: ethylacetate/hexane = 1/2) gave DD–K-182 (11.5 mg, 21.6 μmol, 80.6% yield) as a brownish oil. IR ($CHCl_3$) γ/cm^{-1}: 3405, 3210, 2927, 2854, 1728, 1612, 1454, 763; ^{1}H NMR ($CDCl_3$) δ: 0.87 (t, 3H, J = 6.8 Hz, –CH_2CH_3), 1.03–1.25 (m, 16H, –$(CH_2)_8$–), 1.57–1.70 (m, 2H, $COCH_2CH_2$), 2.27 (t, 2H, J = 7.6 Hz, $COCH_2$), 2.76 (t, 2H, J = 5.6 Hz, NCH_2CH_2O), 3.70 (s, 2H, naph–CH_2N), 3.78 (s, 2H, NCH_2–Ph), 4.18 (t, 2H, J = 5.6 Hz, NCH_2CH_2O), 7.42–7.50 (m, 5H, Ar–H), 7.68–7.82 (m, 6H, Ar–H); ^{13}C NMR ($CDCl_3$) δ: 14.09, 22.66, 24.90, 24.93, 25.58, 29.18, 29.28, 29.32, 29.46, 29.60, 31.89, 33.87, 34.36, 52.04, 58.36, 59.00, 62.08, 125.66, 126.03, 126.87, 126.92, 127.34, 127.62, 127.66, 128.04, 129.01, 129.37, 132.83, 133.30, 136.54, 144.41, 173.78; HR-FAB-MS *m*/*z*: 533.3373 (calcd for $C_{33}H_{45}N_2O_4$, 533.3379).

5.1.7. 1-Cholest-5-en-3-yl 4-{2-[{4-[(hydroxyamino) carbonyl]benzyl}(2-naphthylmethyl) amino]ethyl} succinate (CS–K-182)

To a solution of **2b** (33.0 mg, 36.6 μmol) in THF (1.0 mL) were added acetic acid (2.0 mL) and H_2O (0.5 mL). After refluxing for 7 h, the solution was concentrated in vacuo. Purification of the residue by preparative SiO_2 TLC (eluent: ethylacetate/hexane = 2/3) gave CS–K-182 (18.4 mg, 22.5 μmol, 61.5% yield) as a brownish oil. IR ($CHCl_3$) γ/cm^{-1}: 3421, 2935, 2854, 1728, 1612, 1465, 1365, 1164; ^{1}H

NMR ($CDCl_3$) δ: 0.67 (s, 3H, 18′-CH_3), 0.85 (d, 3H, $J = 1.6$ Hz, 26′ or 27′-CH_3), 0.87 (d, 3H, $J = 1.6$ Hz, 26′ or 27′-CH_3), 0.9–2.1 (m, 26H), 0.91 (d, 3H, $J = 6.4$ Hz, 21′-CH_3), 1.01 (s, 3H, 19′-CH_3), 2.30 (m, 2H, 4′-CH_2), 2.56 (m, 4H, $COCH_2CH_2CO$), 2.74 (m, 2H, NCH_2CH_2), 3.68 (s, 2H, NCH_2–naph), 3.80 (s, 2H, NCH_2Ph), 4.19 (t, 2H, $J = 4.8$ Hz, OCH_2CH_2N), 4.61 (m, 1H, 3′-CH), 5.34 (s, 1H, 6′-CH=C), 7.44–7.54 (m, 5H) 7.69–7.80 (m, 6H); ^{13}C NMR ($CDCl_3$) δ: 11.85, 18.71, 19.28, 21.02, 22.55, 22.80, 23.83, 24.27, 27.72, 28.00, 28.21, 29.18, 29.41, 29.68, 31.85, 31.88, 35.78, 36.19, 36.56, 36.91, 38.03, 39.52, 39.72, 42.32, 49.98, 52.22, 56.15, 56.67, 58.37, 59.26, 62.42, 74.64, 122.77, 125.68, 126.05, 126.94, 127.39, 127.63, 127.67, 128.09, 128.97, 132.84, 133.300, 136.50, 139.50, 144.36, 171.99, 172.15; HR-FAB-MS *m/z*: 819.5304 (calcd for $C_{52}H_{71}N_2O_6$, 819.5234).

5.1.8. 2-[(2-Naphthylmethyl)(4-{[(tetrahydro-2H-pyran-2-yloxy)amino]carbonyl}benzyl)amino] ethyl 2-(2-pyridinyldisulfanyl)ethyl carbonate (**3**)

To a solution of **1** (100 mg, 0.230 mmol) in CH_2Cl_2 (2.0 mL) were added DMAP (167 mg, 1.38 mmol) and triphosgene (23.9 mg, 0.0805 mmol). After being stirred for 15 min at rt, to the solution was added 2-(2-pyridinyldisulfanyl)ethanol [24,29] (43.0 mg, 0.230 mmol) and the solution was stirred for another 7 h at rt. The reaction mixture was diluted with $CHCl_3$ (20 mL), washed with water, dried over Na_2SO_4 and concentrated in vacuo. Purification of the resulting residue by preparative SiO_2 TLC (eluent: ethylacetate/hexane = 3/2) gave **3** (55.0 mg, 36.9% yield) as a colorless oil. IR (neat) γ/cm^{-1}: 3400, 3027, 3010, 2950, 2824, 1743, 1684, 703; ^1H NMR ($CDCl_3$) δ:1.60–1.86 (m, 6H, $CH_2 \times 3$ of THP), 2.79 (t, 2H, $J = 6$ Hz, CH_2CH_2–SS), 3.05 (t, 2H, $J = 3.2$ Hz, NCH_2CH_2), 3.64 (m, 1H, –CH_a–O of THP), 3.72 (s, 2H, NCH_2–naph), 3.80 (s, 2H, $PhCH_2N$), 3.99 (ddd, 1H, $J = 11.2$, 9.2, 2.8 Hz, –CH_b–O of THP), 4.23 (t, 2H, $J = 3.2$ Hz, CH_2CH_2OCO), 4.35 (t, 2H, $J = 6.0$ Hz, OCOO–CH_2–CH_2), 5.08 (m, 1H, OCHO of THP), 7.07–7.81 (m, 15H, Ar–H), 8.45 (m, 1H, Ar–H); ^{13}C NMR ($CDCl_3$) δ: 18.65, 24.99, 28.07, 37.00, 51.79, 58.33, 58.86, 62.61, 65.34, 65.84, 76.68, 77.00, 77.31, 102.60, 119.88, 120.88, 125.63, 125.99, 126.87, 127.24, 127.32, 127.62, 128.04, 128.82 132.78, 133.25, 136.32, 137.05, 143.73, 149.68, 154.76, 159.47; HR-FAB-MS *m/z*: 648.2205 (calcd for $C_{34}H_{38}N_3O_6S_2$ [$M + H^+$], 648.2202).

5.1.9. 2-(Dodecyldisulfanyl)ethyl 2-[(2-naphthylmethyl)(4-{[(tetrahydro-2H-pyran-2-yloxy)amino]carbonyl}benzyl)amino]ethyl carbonate (**4**)

To a solution of **3** (55 mg, 0.085 mmol) in DMSO (1.5 mL) was added dodecanethiol (0.02 mL, 0.085 mmol). After being stirred for 2.5 h at rt, the reaction mixture was diluted with $CHCl_3$ (10 mL), washed with water, dried over Na_2SO_4 and concentrated in vacuo. Purification of the resulting residue by preparative SiO_2 TLC (eluent: ethylacetate/hexane = 3/2) gave **4** (59.0 mg, 93.9% yield) as a colorless oil. IR (neat) γ/cm^{-1}: 3400, 3030, 2927, 2855, 1744, 1683; ^1H NMR ($CDCl_3$) δ: 0.88 (3H, t, $J = 7.2$ Hz, CH_2 $(CH_2)_9CH_3$) 1.15–1.35 (18H, m, $CH_2(CH_2)_9CH_3$), 1.35 (2H, t, $J = 7.2$ Hz, $CH_2(CH_2)_9CH_3$), 1.62–1.86 (6H, m, $CH_2 \times 3$ of THP), 2.69 (2H, t, $J = 7.2$ Hz, SS–$CH_2CH_2(CH_2)_9CH_3$), 2.81 (2H, t, $J = 6.0$ Hz, N–CH_2–CH_2), 2.90 (2H, t, $J = 6.8$ Hz, OCH_2CH_2–SS), 3.64 (1H, m, CH_2CH_a–O of THP), 3.73 (2H, s, NCH_2–naph), 3.80 (2H, s, naph–CH_2N), 4.00 (1H, ddd, $J = 11.2$, 9.2, 2.8 Hz, CH_2CH_b–O of THP), 4.25 (2H, t, $J = 6.0$ Hz, NCH_2CH_2–OCO), 4.34 (2H, t, $J = 6.8$ Hz, $COOCH_2CH_2S$), 5.07 (1H, m, OCHO of THP), 7.45–7.93 (11H, m, Ar–H); ^{13}C NMR ($CDCl_3$) δ: 14.05, 18.68, 22.63, 24.99, 28,06. 28.46, 29.09, 29.16, 29.28, 29.44, 29.53, 29.58, 31.86, 36.82, 39.09, 39.19, 41.18, 51.82, 58.35, 58.91, 60.35, 62.70, 65.81, 65.87, 76.68, 77.00, 77.31, 102.72, 125.61, 125.98, 126.86, 127.22, 127.32, 127.62, 128.04, 128.58, 128.87, 129.71, 130.78, 132.80, 133.27, 136.34, 143.83, 154.87. HR-FAB-MS *m/z*: 738.3816 (calcd for $C_{41}H_{59}N_2O_6S_2$ [$M + H^+$], 739.3815).

5.1.10. 2-(Dodecyldisulfanyl)ethyl 2-[{4-[(hydroxyamino)carbonyl]-benzyl}(2-naphthylmethyl) amino]ethyl carbonate (DDTS–K-182)

To a solution of **4** (29.0 mg, 39.0 μmol) in THF (0.4 mL) were added acetic acid (0.8 mL) and H_2O (0.2 mL). After refluxing for 6 h, the solution was concentrated in vacuo. Purification of the residue by preparative SiO_2 TLC (eluent: ethylacetate/hexane = 1/2) gave DDTS–K-182 (19.0 mg, 76.0% yield) as a brownish oil. IR (neat) γ/cm^{-1}: 3300, 3026, 2928, 2855, 1730, 1653, 705; ^1H NMR ($CDCl_3$) δ: 0.88 (t, 3H, $J = 7.2$ Hz, $CH_2(CH_2)_{10}CH_3$), 1.15–1.45 (m, 20H, $CH_2(CH_2)_{10}CH_3$), 2.69 (t, 2H, $J = 7.2$ Hz, SS–$CH_2(CH_2)_{10}CH_3$), 2.81 (t, 2H, $J = 5.2$ Hz, NCH_2CH_2O), 2.90 (t, 2H, $J = 5.6$ Hz, OCH_2CH_2–SS), 3.73 (s, 2H, NCH_2–naph), 3.80 (s, 2H, $PhCH_2N$), 4.24 (t, 2H, $J = 5.2$ Hz, NCH_2CH_2OCO), 4.34 (t, 2H, $J = 5.6$ Hz, OCH_2CH_2–SS), 7.47–7.81 (m, 11H, Ar–H); ^{13}C NMR ($CDCl_3$) δ: 14.06, 14.14, 20.99, 22.64, 28.47, 29.10, 29.18, 29.30, 29.46, 29.55, 29.59, 29.60, 29.65, 31.88, 36.82, 39.20, 51.91, 58.39, 59.03, 60.41, 65.83, 65.90, 125.66, 126.02, 126.87, 127.37, 127.63, 128.07, 128.99, 132.82, 133.28, 136.32, 144.05, 154.91; HR-FAB-MS *m/z*: 654.3243 (calcd for $C_{36}H_{51}N_2O_5S_2$ [$M + H^+$], 655.3239).

5.2. Preparation of nanoparticles and nanoplexes

OH-Chol was synthesized as previously described [7]. Tween 80 was obtained from NOF Co. Ltd. (Tokyo, Japan). NPs were prepared by a modified ethanol injection method as previously described [7]. For example, in the case of NP–K-182–DD, OH-Chol:Tween 80:K-182–DD = 85:5:10 molar ratio (=10:1.42:1.17, weight) was dissolved in about 5 mL of ethanol, then the ethanol was removed with a rotary evaporator till 1–2 mL was left. Next, a constant volume of water was added to the ethanol solution. NPs formed instantly after further evaporation of the residual ethanol. The concentration of OH-Chol was adjusted to 1 mg/mL in the final NP suspension with drop of water. Then the nanoparticle suspension was filtered through 0.45-μm Millex-HA filters (Millipore, Cork, Ireland) to sterilize it. The particle size distributions were measured by the dynamic light scattering method (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan), at 25 °C after the dispersion was diluted to an appropriate volume with water.

5.3. Cell culture

PC-3 cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). The cells were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and kanamycin (100 μg/mL) at 37 °C in a 5% CO_2 humidified atmosphere.

For the preparation of PC-3 cells stably expressing *Gaussia* luciferase (Gluc), PC-3 cells were plated on 35-mm culture dishes. Twenty-four hours later, the cells were transfected with 2 μg of pCMV-Gluc (New England Biolabs, MA, USA) using lipofectamine 2000 reagents (Invitrogen). The transfected cells were selected in medium with 800 μg/mL G418 sulfate for 2 weeks. G418-resistant colonies were subcultured and established as a permanent cell line transduced with pCMV-Gluc (PC-3-Gluc) and were used for subsequent experiments.

5.4. Transfection

Based on a preliminary experiment [25], the optimized ratio (+/−) of cationic lipid to plasmid DNA was determined as 3:1. The nanoplex at a charge ratio (+/−) of 3/1 was formed by addition of each NP (9.5 μL) to plasmid DNA with gentle shaking and leaving at room temperature for 10 min. For transfection, each nanoplex was

diluted in 1 mL of medium supplemented with 10% FBS and then incubated with the cells for 24 or 48 h.

5.5. Luciferase assay

PC-3 and PC-3-Gluc cells were plated on 96-well culture dishes. For transfection of pCMV-Gluc, each nanoplex of pCMV-Gluc was diluted in 1 mL of medium supplemented with 10% FBS and then incubated with PC-3 cells for 24 h. For transfection of pGL3-basic (Promega, Madison, WI, USA), each nanoplex of pCMV-basic was diluted in 1 mL of medium supplemented with 10% FBS and then incubated with PC-3-Gluc cells for 24 h or 48 h. The level of Gluc activity was evaluated by luciferase activity in the medium, which was measured as counts per sec (cps)/culture medium (mL) using a *Gaussia* Luciferase Assay Kit (New England BioLabs, Inc., MA, USA). Gluc activity (%) was calculated as relative to the Gluc activity (cps/mL) of NP.

5.6. Immunoblotting

PC-3 cells were seeded in a 35-mm culture dish and incubated overnight. The cells at 30% confluency were transfected with nanoplexes containing pGL3-basic in the presence or absence of the HDAC inhibitor and then incubated for 24 h. The cells were suspended in lysis buffer (1% Triton-X 100 in phosphate-buffered saline pH 7.4 (PBS)), and then centrifuged at 15,000 rpm for 10 min. The supernatants (3 μg protein) were resolved on a 12% sodium dodecyl sulfate–polyacrylamide gel by electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (FluoroTrans® W, PALL Gelman Laboratory, Ann Arbor, MI, USA). Acetylated histone H3 was detected by rabbit anti-human acetyl histone H3 antibody (Sigma Chemical Co., St. Louis, MO, USA). The goat anti-rabbit IgG peroxidase conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as secondary antibody. These proteins were detected with peroxidase-induced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA).

5.7. Cytotoxicity

Cytotoxicity upon transfection using NP–K-182–DD was evaluated with a cell proliferation assay kit (Dojindo, Kumamoto, Japan). PC-3 cells were placed in a 96-well plate in medium containing 10% FBS, and were transfected at various concentrations of nanoplex. After 24 h of incubation, the medium was removed, and the cells were treated with a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) solution (10 μL) in medium containing serum (100 μL) for 30 min. Cell viability was expressed as relative to the absorbance at 450 nm of untransfected cells.

5.8. Evaluation of histone deacetylase inhibitory activities

The IC_{50} values were measured by using CycLex HDAC Assay Kit (CycLex Co. Ltd, Nagano, Japan) according to the manufacturer's protocol. The IC_{50} values represent the molar concentrations (μM) required to inhibit the HDACs by 50%.

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