Design and synthesis of prostate cancer antigen-1 (PCA-1/ALKBH3) inhibitors as anti-prostate cancer drugs

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Key words; PCA-1, ALKBH3, Inhibitor, Anti-prostate cancer drug, Small compound, Bioavailability.

Abstract

A series of 1-aryl-3,4-substituted-1*H*-pyrazol-5-ol derivatives was synthesized and evaluated as prostate cancer antigen-1 (PCA-1/ALKBH3) inhibitors to obtain a novel anti-prostate cancer drug. After modifying 1-(1*H*-benzimidazol-2-yl)-3,4-dimethyl-1*H*-pyrazol-5-ol (**1**), a hit compound found during random screening using a recombinant PCA-1/ALKBH3, 1-(1*H*-5-methylbenzimidazol-2-yl)-4-benzyl-3-methyl-1*H*-pyrazol-5-ol (**35**, HUHS015), was obtained as a potent PCA-1/ALKBH3 inhibitor both *in vitro* and *in vivo*. The bioavailability (BA) of **35** was 7.2% in rats after oral administration. As expected, continuously administering **35** significantly suppressed the growth of DU145 cells, which are human hormone-independent prostate cancer cells, in a mouse xenograft model without untoward effects.

Prostate cancer is highly serious when malignant, and developing novel anti-prostate cancer drugs that are effective against both androgen-dependent and independent types is now urgently required.[1] Tsujikawa *et al.* have reported a novel gene that encodes a DNA and/or RNA-alkylating damage-repair enzyme called prostate cancer antigen (PCA)-1[2] or AlkB homologue 3 (ALKBH3) that is highly expressed in clinical prostate cancer cells; genetic inhibition of the enzymatic activity by injecting siRNA effectively inhibited the growth of androgen-independent prostate cancer cells, such as DU145, that express high levels of PCA-1/ALKBH3.[3, 4] In addition, losing PCA-1/ALKBH3 led to

3-methylcytosine accumulation and reduced cell proliferation in various cell lines.[5] Therefore, a small, orally available PCA-1/ALKBH3 inhibitor would be a novel and clinically effective anti-prostate cancer drug, even for hormone-independent varieties. Random screening using commercially available 17,000-compound libraries and recombinant PCA-1/ALKBH3 has been carried out to identify small PCA-1/ALKBH3 inhibitors; 1-(1*H*-benzimidazol-2-yl)-3,4-dimethyl-1*H*-pyrazol-5-ol (**1**) is a hit compound with 61% inhibition at 10 μM, and its inhibitory activity was confirmed with 76% inhibition at the same concentration using re-synthesized **1** (Fig. 1). However, **1** demonstrated only a 0.8% inhibitory effect against the proliferation of DU145 cells at 10 μM. Accordingly, we focused our efforts to obtain a novel PCA-1/ALKBH3 inhibitor that is effective in cell assays and *in vivo*.

We report the design and synthesis of novel 1-ary-3,4-substituted-1*H*-pyrazol-5-ol derivatives and their PCA-1/ALKBH3 inhibitory activities and anti-cancer effects *in vivo*. Among them, 1-(5-methyl-1*H*-benzimidazol-2-yl)-4-benzyl-3-methyl-1*H*-pyrazol-5-ol (**35**, HUHS015) was a potent PCA-1/ALKBH3 inhibitor that significantly suppressed the growth of DU145 cells *in vitro* and in a mouse xenograft model. These small PCA-1/ALKBH3 inhibitors are highly important because PCA-1/ALKBH3 is associated with numerous cancers, such as lung[5, 6] and pancreatic cancers.[5, 7]

Figure 1

The substituted pyrazoles evaluated in this work were synthesized by condensing N-substituted hydrazines (**3**) with ethyl acetoacetate derivatives (**4**)[8] (Scheme 1). The assay methods for PCA-1 inhibitory activities and biological assays *in vitro* and *in vivo* of these compounds were shown in Supplementary data.

Scheme 1

The deletion and replacement of the aromatic rings on the benzimidazole portion of **1** generated only weak anti-PCA-1/ALKBH3 activities, indicating the importance of the benzimidazole (**5**-**13**, Table 1). Only compounds with a methyl group at the 5 position of benzimidazole (**12**) demonstrated slightly more potent anti-PCA-1/ALKBH3 activity. Structurally similar 1-aryl-3,4-substituted-pyrazol-5-ol compounds, such as the 1-(2-pyridyl)-pyrazol-5-ol derivative (**2**), were recently reported to block divalent metal transporter 1 (DMT1, Fig. 1).[9] In these DMT1 blockers, the neighboring basic nitrogen in the aryl ring was vital for metal chelation and DMT1 inhibitory activities. However, a similar 2-pyridyl derivative (**11**) examined in this study revealed only weak PCA-1/ALKBH3 inhibition and attenuated inhibitory activities against DU145 cell proliferation (data not shown). The basicity of the N atom on the benzimidazole studied in this work is weak, and its metal chelating ability should be poor. Therefore, we proposed that metal chelation contributed little to the PCA-1/ALKBH3 inhibitory activities. This result was favorable for developing a clinically useful anti-cancer drug because metal chelators often cause adverse effects or toxicity *in vivo*.

Table 1 and Table 2

Based on the above results, we fixed 1*H*-benzimidazol-2-yl at the 1 position of the pyrazole ring and evaluated 1-(substituted-1*H*-benzimidazole-2-yl)-3,4-substituted-1*H*-pyrazol-5-ol derivatives (**14**-**24**, Table 1). Introducing a phenyl (**15**) or naphthyl (**16**) group on the methyl group at the 4 position of the pyrazole generated potent PCA-1/ALKBH3 inhibitory effects, while the compounds without the methyl (**14**) group or with a carboxyl moiety (**17** and **18**) demonstrated similar activities. Derivatives with a phenyl ring at the 3 position (**19**-**24**) exhibited weaker activities. Accordingly, we fixed the methyl and benzyl groups at the 3 and 4 positions, respectively, for further studies.

The substituent effects on the phenyl group in **15** were evaluated (**25**-**34**, Table 2). However, no enhancement of anti-PCA-1/ALKBH3 activities was observed. In particular, the derivative with an *ortho*-chloro substituent (**25** and **28**) greatly decreased the inhibitory activity. Therefore, a benzyl group was used at the 4 position, and the derivatives bearing benzimidazole ring substituents were evaluated (**35**-**44**, Table 2). Some derivatives with substitution at the 5 position, such as **35** and **37**-**40**, exhibited potent PCA-1/ALKBH3 inhibitory activity, while compounds with substituents at the 4 position, such as **43** and **44**, displayed reduced activity. Of these compounds, **35** (HUHS015) was selected for further study because it potently inhibited PCA-1/ALKBH3 and had good physical properties. Finally, the substituents on the phenyl ring of **35** were varied (**45**-**52**, Table 2). The inhibitory activities of those derivatives

were similar to that of **35** and therefore were not pursued.

Next, we selected several compounds and tested the proliferation inhibition against DU145 cells and the oral availability in rats (Table 3). The proliferation inhibition of DU145 was examined in both an ordinary anchorage-dependent assay on dishes and an anchorage-independent assay using soft-agar because the inhibitory activity against the latter cells might be crucial to clinical efficacy.[10] Compounds with weak PCA-1/ALKBH3 inhibitory activity, such as **12**, demonstrated weak proliferation inhibition against DU145, while those with sub-micromolar IC_{50} values, such as **15**, **35**, **38**, and **49**, exhibited more potent effects. We are uncertain about the potency of **36** in the DU145 assays because its IC_{50} value during the PCA-1/ALKBH3 enzyme assay was 4.5 μM or weaker. Consequently, **36** was eliminated from further studies because these inconsistent effects might cause unexpected side effects in future studies. To select a compound for additional *in vivo* studies, we also measured the blood concentration 1 h after each compound was orally administered (32 mg/kg). Compound **12** exhibited high serum concentrations relative to the other derivatives. However, compounds with relatively high molecular weights, such as **41**, exhibited poor oral absorbability. Therefore, smaller compounds were more bioavailable. Consequently, we selected **35** (HUHS015) for further studies because it exhibited an adequate level of serum concentration after oral administration and potent inhibition during both the enzymatic and cell assays. We measured the preliminary bioavailability (BA) value for **35** after oral administration to rats, revealing a 7.2% BA value (Figs. S1 and S2). This BA value was sufficient for continuing the *in vivo* studies.

Table 3 and Figure 2

The growth inhibition demonstrated by **35** (32 mg/kg) was examined in a mouse xenograft model bearing DU145 after subcutaneous injection (Fig. 2); potent growth inhibition was observed without limiting weight gains, even after a 6-day continuous administration.[11] Therefore, the synthesizing small molecule inhibitor of PCA-1/ALKBH3, a clinically identified novel target, demonstrated promising results for developing an anti-prostate cancer drug without mechanism-oriented side effects. Now further modifications of **35** to obtain more potent inhibitors and combination studies with **35** and clinically used drug such as docetaxel are in progress, and will be reported in the near future.

In conclusion, we synthesized 1,3,4-substituted-1*H*-pyrazol-5-ol derivatives to identify orally active PCA-1/ALKBH3 inhibitors and selected 1-(5-methyl-1*H*-benzimidazol-2-yl)-4-benzyl-3-methylpyrazol-5-ol (**35**, HUHS015) for further analysis. Compound **35** exhibited potent inhibition against DU145 proliferation without observable side effects after subcutaneous administration in a xenografted mouse model. The results for **35** (HUHS015) in the xenograft mouse model, when combined with previously reported PCA-1/ALKBH3 knock-out mice study,[12] in which mice lacking function of ALKBH3 gene are viable without overt phenotypes and histological changes, demonstrated that small PCA-1/ALKBH3 inhibitors would be effective drugs against prostate cancer without mechanism-based side effects or toxicity.

Acknowledgments

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Supplementary data

The supplementary data (experimental procedures, Table S1, and Figures S1-S2) are available.

References and Notes

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8. The representative procedure for synthesizing the compounds studied in this work is as follows: A mixture of 2-hydrazino-5-methyl-benzimidazole (3, 1.0 g, 6.17 mmol) and ethyl 2-acetyl-3-phenylpropanoate (4, 1.4 mL, 6.59 mmol) in acetic acid (20 mL) was stirred for 2 h at ambient temperatures. To a mixture of acetonitrile (100 mL) and water (100 mL) was added the reaction mixture. After stirring at ambient temperatures, the resulting precipitates were collected by filtration and washed with acetonitrile in water (1:1). The precipitates was purified by recrystallization

from ethanol (95 mL) to generate 3-methyl-1-(5-methyl-1H-benzimidazol-2-yl)-4-benzyl-1H-pyrazol-5-ol (35, 0.64 g, 32.6%). The other compounds studied in this work (5–52) were prepared in a similar manner. The typical experimental procedure for synthesizing and evaluating the compounds used in this study, in addition to the analytical data and the estimated purity (HPLC) of the biologically relevant compounds, are provided in Supplemental data and Table S1, respectively.

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10. (a) Hamburger, A. W.; Salmon, S. E. Science 1977, 197, 461; (b) Williams, T. J.; Lieber, M. M.; Podratz, K. C.; Malkasian, G. D., Jr. Am. J. Obstet. Gynecol. 1983, 145, 940.

11. No abnormal finding on mice's behavior and organs has been observed even after a 6-days continuous administration.

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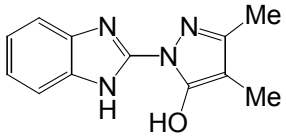
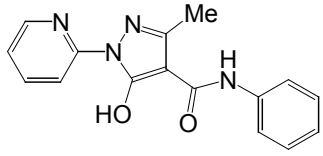
Figure 1.



Hit compound (**1**)



DMT1 blocker (**2**)

Figure 1. Structure of PCA-1/ALKBH3 inhibitor obtained in our random screening and previously known DMT1 blocker.

Figure 2

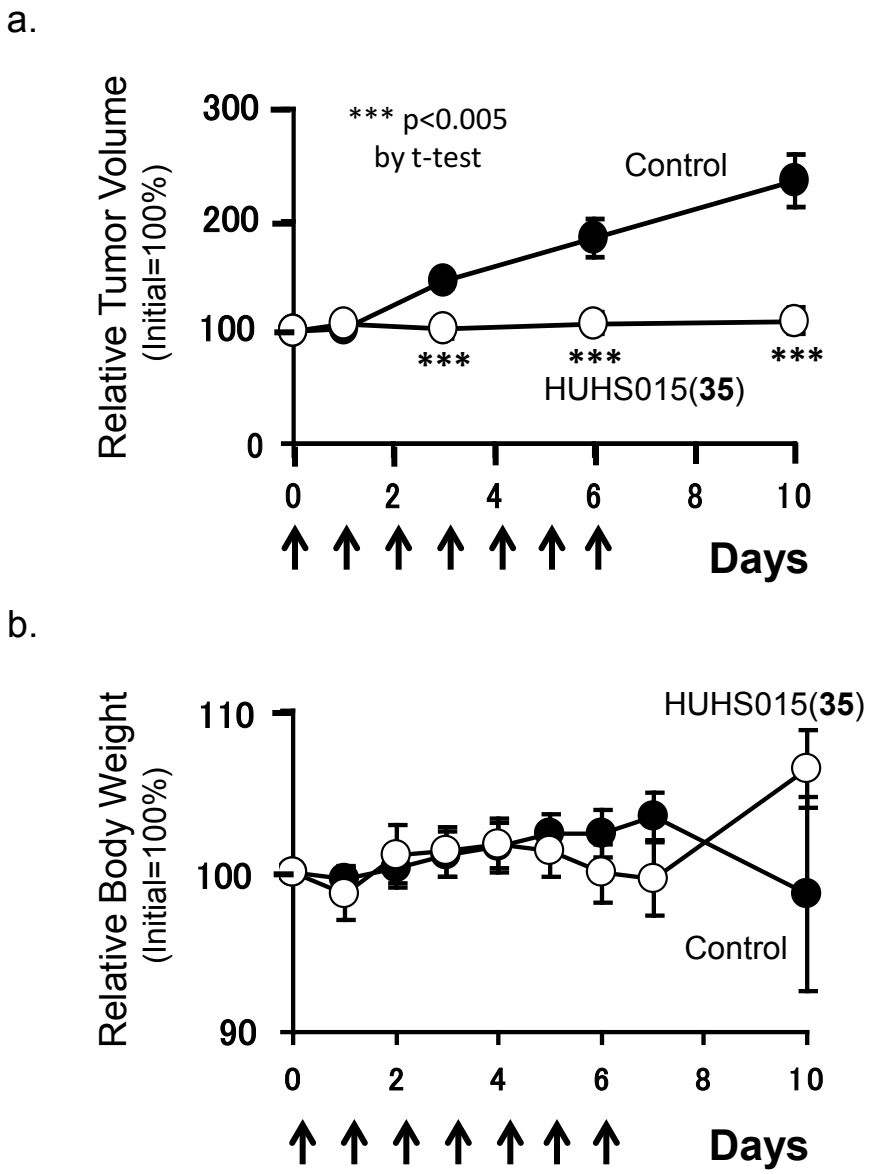
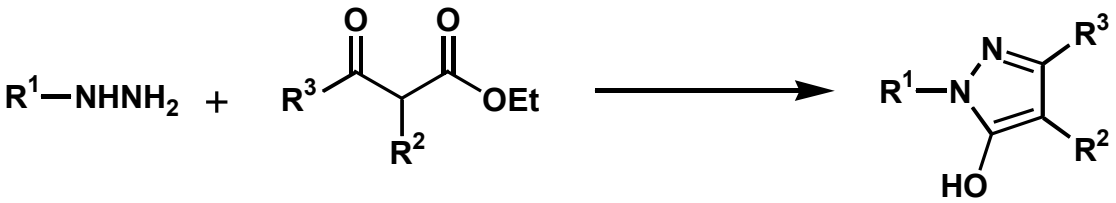


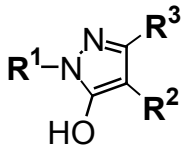
Figure 2. **(**a) Inhibitory effect of HUHS015 (**35**, 32 mg/kg/day, open circle) on the growth of subcutaneously implanted DU145 (nude mice) was examined (n = 6). Compound **35** or saline was administered for 6 days (arrows), and the tumor volume was measured for 10 days. Black circles demarcate the control data (0.5% methylcellulose). (b) The mice administered with **35** (white circle) gained more body weight than the control mice (black circle). No apparent toxicity or side effects were found in the mice during the experiment. The values represent means ± SE (n = 6).

Scheme 1.



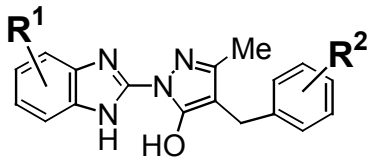
3 **4** **5-52**

Table 1. PCA-1 inhibitory activities of 1,3,4-substituted-5-hydroxy-pyrazoles



	R^1	R^2	R^3	PCA-1 inhibition IC_{50} (μM)
5	Bezimidazol-2-yl	Me	Me	4.0
6	Phenyl	Me	Me	>10 (20%)
7	Benzthiazol-2-yl	Me	Me	>10 (-3%)
8	Benzoxazol-2-yl	Me	Me	>10 (0%)
9	1-Me-benzimidazol-2-yl	Me	Me	>10 (21%)
10	2-Pyrimidyl	Me	Me	>10 (-4%)
11	2-Pyridyl	Me	Me	>10 (-4%)
12	5-Me-benzimidazol-2-yl	Me	Me	2.6
13	4-Me-benzimidazol-2-yl	Me	Me	>10 (-28%)
14	Benzimidazol-2-yl	H	Me	3.6
15	Benzimidazol-2-yl	Benzyl	Me	0.48
16	Benzimidazol-2-yl	2-Naphtylmethyl	Me	0.75
17	Benzimidazol-2-yl	CH_2COOMe	Me	4.3
18	Benzimidazol-2-yl	CH_2COOH	Me	15.0
19	Benzimidazol-2-yl	H	Phenyl	7.2
20	Benzimidazol-2-yl	Me	Phenyl	>10 (-11%)
21	Benzimidazol-2-yl	Benzyl	Phenyl	>10 (2%)
22	5-Me-benzimidazol-2-yl	Me	Phenyl	3.3
23	5-Cl-benzimidazol-2-yl	Phenyl	Phenyl	2.0
24	5-Me-benzimidazol-2-yl	Phenyl	Phenyl	5.8

Table 2. PCA-1 inhibitory activities of 1-(substituted benzimidazol-2-yl)-5-hydroxy-3-methyl-4-(subsituted benzyl) pyrazoles



	R^1	R^2	PCA-1 inhibition IC_{50} (μM)
25	H	2-Cl	>10 (-13%)
26	H	3-Cl	0.54
27	H	4-Cl	>10 (46%)
28	H	2,4-Cl_2	>10 (11%)
29	H	3,4-Cl_2	3.1
30	H	4-CF_3	0.55
31	H	4-F	0.60
32	H	4-MeO	0.63
33	H	4-Phenyl	0.72
34	H	4-tBu	4.1
35	5-Me	H	0.67
36	5-Cl	H	4.5
37	5-Br	H	0.49
38	5-CF_3	H	0.61
39	5-tBu	H	0.64
40	5-Phenyl	H	0.81
41	5-(4-Cl-phenyl)	H	1.5
42	5-(4-Me-phenyl)	H	2.1
43	4-Me	H	5.9
44	4,5-Me_2	H	>10 (-47%)

Table 2. (continued)

	R^1	R^2	PCA-1 inhibition IC_{50} (μM)
45	5-Me	4-CF_3	0.57
46	5-Me	4-MeO	0.68
47	5-Me	3,4-Cl_2	0.72
48	5-Me	2-Cl	0.91
49	5-Me	4-Cl	0.75
50	5-Me	4-F	0.78
51	5-Me	4-tBu	3.7
52	5-Me	4-Phenyl	5.3

Table 3. Inhibitory activities of DU145 cells proliferation on dish (2-D assay) and in agar (3-D assay), and serum concentration at 1 h after oral administration (32 mg/kg).

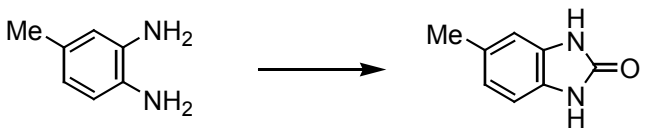
	Inhibition (%) on DU145 proliferation				Serum concentration at 1 h (μg/mL)
	2D assay		3D assay		
	10 μM(%)	1 μM(%)	10 μM(%)	1 μM(%)	
15	47	42	90	42	NT[a]
12	19	-11	69	3.8	1080
35	54	35	81	34	81
36	*NT*	86	87	84	154
38	84	78	92	90	20
41	63	70	100	91	7
49	46	-1	90	2.8	72

[a] NT: not tested.

Supplemental experiment data

Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F254 plate. For normal chromatography, Merck silica gel type 60 (size 5-40 μm) was used. All evaporation was performed with a rotary evaporator under reduced atmosphere. The structures of all compounds were confirmed by a LC-MS (Brucker, micrOTOF-Q), a FT-IR(JEOL, FT/IR-4100), and 400MHz proton nuclear magnetic resonance spectrum (JEOL, ECX-400PKS). The chemical shift values are reported in parts per million on the δ scale from internal standard tetramethylsilane. No attempt was made to maximize the yields. Purity of the synthesized compounds was determined using the HPLC method with UV detector at the absorption wavelength of 254 nm. Purity of all biologically estimated compounds was ≥95%.

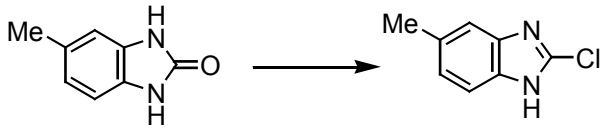
Typical experimental procedure for substituted 1,3-dihydrobenzimidazol-2-one



To a solution of 4-methyl-1,2-phenylenediamine (25 g, 0.205 mol) in tetrahydrofuran (375 mL) was added dropwise a solution of 1,1'-carbonyldiimidazole (36.5 g, 0.225 mol) in dichloromethane (375 mL). After stirring for 6.5 h at ambient

temperature, to the reaction mixture was added diisopropyl ether (375 mL). After stirring at ambient temperature, the resulting precipitates were collected by filtration. The precipitates were washed with diisopropyl ether and dried *in vacuo* to give 5-methyl-1,3-dihydrobenzimidazol-2-one (24.6 g, 70.1%). ESI-HRMS calcd. for $C_8H_8N_2ONa([M+Na]^+)$ 171.0529; found m/z 171.0529. NMR (DMSO-d_6, δ): 2.27(3 H, s), 6.70-6.81(3 H, m), 10.46(2 H, br. s).

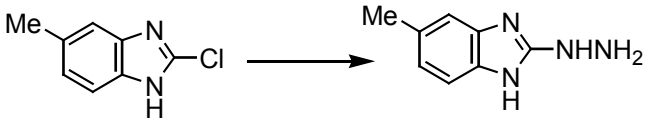
Typical experimental procedure for substituted 2-chloro-benzimidazol



A mixture of 5-methyl-1,3-dihydrobenzimidazol-2-one (1.00 g, 6.84 mmol) and phosphorous oxychloride (9.54 mL, 103 mmol) was stirred for 1.5 h at 95 ^oC. After being cooled to ambient temperature, the reaction mixture was carefully added to a mixture of saturated $NaHCO_3$ aq. (60 mL) and ethyl acetate (60 mL). The separated organic layer was washed with water, brine, and dried over $MgSO_4$. After filtration, the filtrate was evaporated *in vacuo*, The resulting precipitates were collected by filtration, and successfully washed with isopropyl ether to give 2-chloro-5-methyl-benzimidazole (1.74 g, 58.9 %). ESI-HRMS calcd. for $C_8H_8ClN_2$ $([M+H]^+)$ 167.0371; found m/z 167.039. NMR (DMSO-d_6, δ): 2.40 (3 H, s),

7.00-7.06 (1H,m), 7.29 (1 H, s), 7.39 (1 H, d, J=8.2 Hz). Other substituted 2-chloro-benzimidazols were prepared in a manner similar.

Typical experimental procedure for substituted 2-hydrazino-benzimidazol (3)



A mixture of 2-chloro-5-methyl-benzimidazole (10.2 g, 61.2 mmol) and hydrazine monohydrate (59 mL, 1.22 mol) was stirred at 100 ^{o}C overnight. After being cooled to ambient temperature, to the reaction mixture was added to water (60 mL). After stirring under ice cooling, the resulting precipitates were collected by filtration. The precipitates were washed with water 3 times, and then dried *in vacuo* to give 2-hydrazino-5-methyl-benzimidazole (8.4 g, 84.6%). ESI-HRMS calcd. for $C_8H_{11}N_4([M+H]^+)$ 163.0978; found m/z 163.0985. NMR (DMSO-d_6, δ): 2.30 (3 H, s), 4.39 (2 H, br. s), 6.63-6.70 (1 H, m), 6.91-6.94 (1 H, m), 6.97-7.01 (1 H, m), 7.69 (1 H, br. s), 10.87 (1 H, br. s). Other substituted hydrazine derivatives (**3**) were prepared in a similar manner.

Cell culture. The human prostate cancer cell line DU145 was supplied from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University, Japan.. The cell line was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 μg/mL kanamycin at 37°C under a humidified atmosphere containing 5% CO_2.

Animal care. Male nude mice of BALB/c background were purchased from Charles River Japan Inc., Tokyo. Male Sprague-Dawley (SD) rats were purchased from Japan SLC Inc. (Shizuoka, Japan). Animals were kept under conditions of constant temperature and humidity, and fed a standard diet and water ad libitum. All animal experiments in this study were approved by our institutional animal care committee.

PCA-1 inhibitory activity. An assay system of PCA-1 demethylase inhibitors was described elsewhere. Briefly, 4 ng of PCA-1 with or without compounds was incubated with 80 fmol 3-methyl cytosine oligo DNA (100 bp) as a substrate to be demethylated in buffer (50 mM Tris-HCl (pH8.0), 2 mM ascorbic acid, 100 μM oxoglutarate, and 40 μM ferrous sulfate) and incubated at 37°C for 1 hour. Then, the reaction was stopped by dilution to 20 times volume by water. Two μL of the above samples was used as templates of real-time PCR using Bio-Rad iQ SYBR Green Supermix to 20 μL. Standard curve was given using oligo DNA without methylation as templates. The

cycling conditions consisted of an initial, single cycle at 95°C for 10 sec followed by 40 cycles of at 95°C for 5 sec, at 61°C for 30 sec, and at 72°C for 15 sec. The PCA-1 inhibition effects of compounds were determined by quantitative analysis with real-time PCR.

Inhibitory effect of compounds on proliferation of DU145 by anchorage-dependent (on dish) cell culture. The experiments to examine the inhibitory effects of compounds with DU145 cells proliferation were carried out in a 96-well plate and the number of viable cells at the end of incubation was determined by WST ((2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)) assay, which is determined by measuring the capacity of the cells to reduce WST to formazan. Briefly, 5 x 10^3 cells / well in 90 μL cultured medium were incubated in the presence or absence of compounds for 48 hours. After addition of 10 μL of WST-1/1-methoxy-5-methylphenazinium methylsulfate solution, the plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 hours. Absorbance was measured using a microplate reader with a test wavelength of 450 nm and a reference wavelength of 630 nm. Then, the percent of inhibition of each compound was calculated.

Inhibitory effect of compounds on proliferation of DU145 by anchorage-independent (in agar, 3D assay) cell culture. Inhibitory effects of the compounds on proliferation of DU145 cells in 3D cell culture were examined by assaying colony formation in soft agar. Briefly, DU145 cells were suspended to 1.3 x 10^5 cells /mL in DMEM with 0.4% Agarose, 10 % FCS and antibiotics, then, 75 μL of that containing 1×10^4 cells were plated on the bottom layer that consisted with 0.6% agarose containing DMEM 10 % FCS in 96 well plates. The plates were placed at 4°C for 5 min to harden and warmed at 37°C in a humidified atmosphere containing 5% CO_2. Hundred μL of the compound solutions were added on the each well. The cell numbers after 7 days were determined with the capacity of the cells to reduce 12 μL of 5 mM WST to formazan at 37 °C for 2 hours by measurement of absorbance at 450 nm. Percentages of inhibition of each compound were calculated.

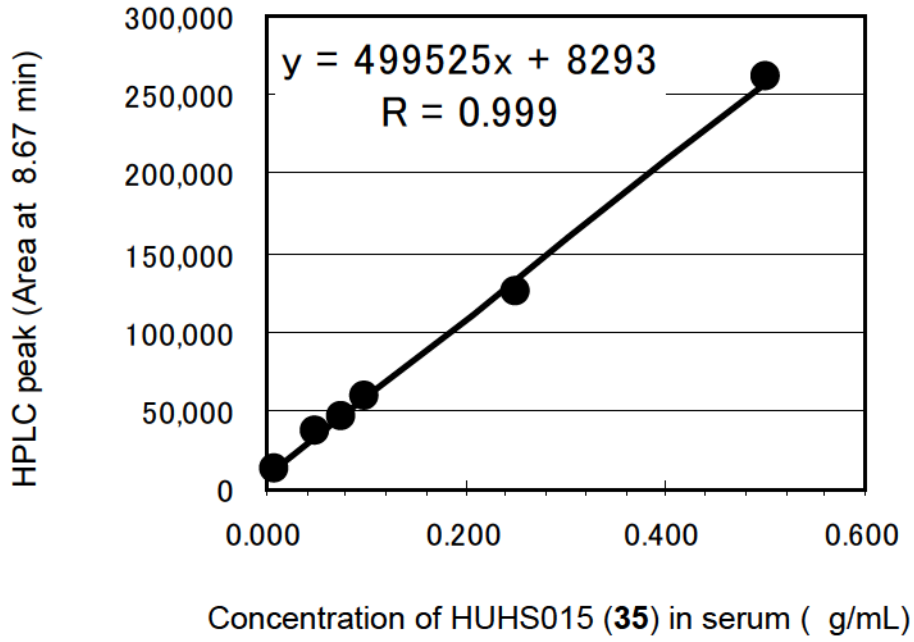
Measurement of serum concentration of compounds in rats Male Sprague-Dawley (SD) rats were fasted for 18 hours, and 32 mg/kg of each compound suspended in 0.5 % methylcellurose (MC) was orally administrated. After 1 hour of administration, bloods were obtained with heparin as anticoagulant from abdominal vein or subclavian vein under anesthesia. The serum was then separated by

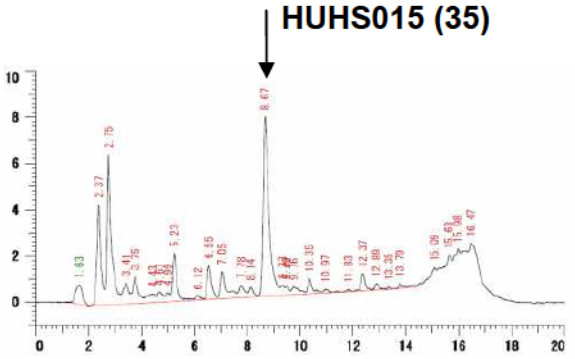
centrifugation and all samples were stored at -30°C until analyzed. The concentration of each compound was determined using HPLC.

Measurement of bioavailability of compounds in rats Male Sprague-Dawley (SD) rats were fasted for 18 hours, and 32 mg/kg of HUHS15 suspended in 0.5 % methylcellurose (MC) was orally or 0.5 mg/kg of HUHS15 dissolved in Sesame oil was intravenously administrated. After 0.25, 0.5, 1, 2, 6, 24 hours of administration, bloods were obtained from abdominal vein or subclavian vein under anesthesia. The concentration of each compound in the serum was measured using HPLC (Figure S1).

Growth inhibitory effect of 35(HUHS15) on DU145 xenograft in mice. Male nude mice of BALB/c background were implanted subcutaneously with approximately 10 mm^3 of in vivo passage tumors of DU145 cells into the right flank of the mice. When estimated tumor volume in the mice had reached 100 to 300 mm^3, animals were divided into 2 experimental groups of 6 mice and treated subcutaneously with 32 mg/kg of HUHS15 suspended in 0.5 % MC for 6 days everyday. The tumor volume was calculated on day 0, 1, 3, 6, and 10 from the following formula: tumor volume (mm^3) = $L x W^2/2$, where L and W represent the length and the width of the tumor mass, respectively. The changes of tumor volume before administration were calculated.

Results are shown as means ± SE of 6 mice and analyzed statistical significance by t-test.





HPLC conditions

- flow : 1.0 ml/min
- detection at 298 nm
- column :YMC-Pack Pro C18(150 x 4.6 mm, 5 m)
- gradient program

0	1	10	12	12.1	min
35	35	60	99	35	% AN in 0.1% TFA aq. (AN including 0.1% TFA)

AN:acetonitrile

Figure S1. A standard curves between 0.01 and 0.5 g/mL of HUHS015(**35**) were prepared in serum, giving correlation coefficient of 0.999.

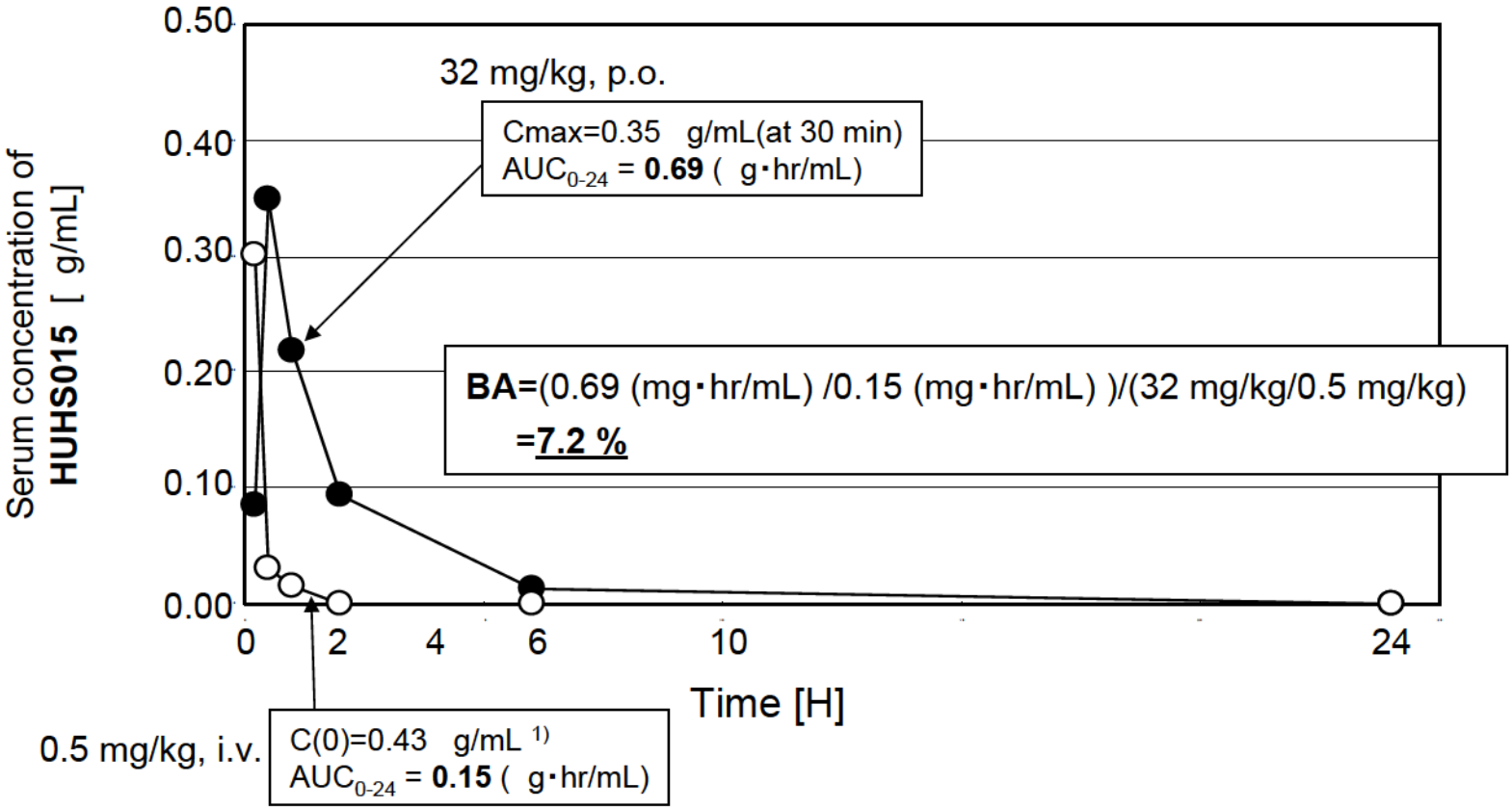


Figure S2. Measurement of preliminary bioavailability (BA) value of HUHS015(35) in rat (n=1).
Sesame oil and 0.5% MC were used as solvent for *i.v.* and *p.o.* studies, respectively. Linearity between AUC at 32 mg/kg (*p.o.*) and that at 10 mg/kg (*p.o.*) was observed (data not shown). C(0) after *i.v.* administration was estimated by a free software (http://www.pharm.kyoto-u.ac.jp/byoyaku/Kinetics/download.html).

Tbale S1. Analytical data of biologically estimated compounds.

	^{1}H-NMR	IR	ESI-HRMS	HPLC[*)]	
				retention time (min)	estimated purity (%)
5	NMR (DMSO-d_6, δ): 1.77 (3H, s), 2.17 (3H, s), 7.12-7.18 (2H, m), 7.50-7.55 (2H, m)	IR (KBr): 3296, 3049, 2921, 2862, 1626, 1547 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{12}H_{13}N_4O$ ($[M+H]^+$) 229.1084 ; found 229.1091	8.5	98.3
6	NMR (DMSO-d_6, δ): 1.76 (3H, s), 2.09 (3H, s), 7.14-7.20 (1H, m), 7.38-7.44 (2H, m), 7.71 (2H, d, *J* = 7.8Hz)	IR (KBr): 3431, 3046, 2859, 2796, 1591 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{11}H_{13}N_2O$ ($[M+H]^+$) 189.1022 ; found 189.1035	8.6	96.7
7	NMR (DMSO-d_6,δ): 1.76 (3H, s), 2.20 (3H, s), 7.33-7.38 (1H, m), 7.46-7.51 (1H, m), 7.80-7.84 (1H, m), 8.03-8.07 (1H, m)	IR (KBr): 3067, 2924, 1644, 1596, 1528 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{12}H_{12}N_3OS$ ($[M+H]^+$) 246.0696 ; found 246.0710	11.3	98.3
8	NMR (DMSO-d_6,δ): 1.77 (3H, s), 2.15 (3H, s), 7.32-7.41 (2H, m), 7.65-7.75 (2H, m)	IR (KBr): 3082, 2919, 1633, 1609, 1561 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{12}H_{12}N_3O_2$ ($[M+H]^+$) 230.0924 ; found 230.0930	9.9	98.8
9	NMR ($CDCl_3$,δ): 1.93 (3H, s), 2.22 (3H, s), 4.24 (3H, s), 7.26-7.36 (3H, m), 7.56-7.61 (1H, m)	IR (KBr): 3431, 3058, 2922, 2861, 1665, 1561, 1511 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{13}H_{15}N_4O$ ($[M+H]^+$) 243.1240 ; found 243.1248	8.7	98.6
10	NMR (DMSO-d_6,δ): 1.73 (3H, s), 2.11 (3H, s), 7.31 (1H, t, *J* = 4.8Hz), 8.77 (2H, d, *J* = 4.8Hz)	IR (KBr): 3476, 3056, 2927, 1639, 1568 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_9H_{11}N_4O$ ($[M+H]^+$) 191.0927 ; found 191.0934	7.3	99.0
11	NMR (DMSO-d_6,δ): 1.72 (3H, s), 2.12 (3H, s), 7.17-7.22 (1H, m), 7.86-7.92 (1H, m), 8.28 (1H, br s), 8.37-8.40 (1H, m)	IR (KBr): 3419, 3062, 2919, 1633, 1594 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{10}H_{12}N_3O$ ($[M+H]^+$) 190.0975 ; found 190.0979	9.1	98.8
12	NMR (DMSO-d_6, δ): 1.77 (3H, s), 2.16 (3H, s), 2.39 (3H, s), 6.98 (1H, dd, *J* = 0.9 and 8.2Hz), 7.32 (1H, br s), 7.40 (1H, d, *J* = 8.2Hz)	IR (KBr): 3308, 3018, 2920, 2861, 1635, 1573 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{13}H_{15}N_4O$ ($[M+H]^+$) 243.1240 ; found 243.1259	9.0	98.9
13	NMR (DMSO-d_6,δ): 1.78 (3H, s), 2.18 (3H, s), 2.52 (3H, s), 6.94-6.98 (1H, m), 7.05 (1H, t, *J* = 7.8Hz), 7.35 (1H, d, *J* = 7.8Hz)	IR (KBr): 3243, 2912, 1672, 1606, 1497 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{13}H_{14}N_4ONa$ ($[M+Na]^+$) 265.1060; found 265.1062	9.2	98.3
14	NMR (DMSO-d_6, δ): 2.20 (3H, s), 5.24 (1H, s), 7.14-7.20 (2H ,m), 7.49-7.55 (2H, m)	IR (KBr): 3310, 3043, 2905, 1626, 1559 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{11}H_{11}N_4O$ ($[M+H]^+$) 215.0927 ; found 215.0933	7.3	98.6
15	NMR (DMSO-d_6, δ): 2.17 (3H, s), 3.60 (2H, s), 7.13-7.19 (3H, m), 7.24-7.30 (4H, m), 7.49-7.55 (2H, m)	IR (KBr): 3269, 3026, 1627, 1542 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{18}H_{17}N_4O$ ($[M+H]^+$) 305.1397 ; found 305.1396	11.0	99.0
16	NMR (DMSO-d_6,δ): 2.19 (3H, s), 3.77 (2H, s), 7.13-7.18 (2H, m), 7.40-7.55 (5H, m), 7.73 (1H, br s), 7.80-7.86 (3H, m)	IR (KBr): 3068, 2923, 1643, 1596, 1528 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{22}H_{19}N_4O$ ($[M+H]^+$) 355.1553 ; found 355.1577	12.1	99.1
17	NMR (MeOH-d_4,δ): 2.23 (3H, s), 3.39 (2H, s), 3.70 (3H, s), 7.23-7.29 (2H, m), 7.51-7.58 (2H, m)	IR (KBr): 2997, 2950, 1734,1690, 1606, 1500 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{14}H_{15}N_4O_3$ ($[M+H]^+$) 287.1139 ; found 287.1145	8.7	97.5
18	NMR (DMSO-d_6,δ): 2.15 (3H, s), 3.22 (2H, m), 7.13-7.18 (2H, m), 7.49-7.55 (2H, m)	IR (KBr): 3433, 2993, 1690, 1609, 1525 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{13}H_{13}N_4O_3$ ($[M+H]^+$) 273.0982 ; found 273.0967	7.9	97.0

19	NMR (DMSO-d_6,δ): 5.94 (1H, s), 7.22-7.28 (2H, m), 7.39-7.51 (3H, m), 7.55-7.61 (2H, m), 7.88-7.92 (2H, m)	IR (KBr): 3334, 3068, 1678, 1631, 1600, 1555 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{16}H_{13}N_4O$ ($[M+H]^+$) 277.1084 ; found 277.1093	10.0	98.7
20	NMR (DMSO-d_6,δ): 2.06 (3H, s), 7.18-7.24 (2H, m), 7.46-7.60 (5H, m), 7.73-7.78 (2H, m)	IR (KBr): 3173, 1665, 1640, 1562 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{17}H_{15}N_4O$ ($[M+H]^+$) 291.1240 ; found 291.1236	11.1	98.1
21	NMR (DMSO-d_6, δ): 3.84 (2H, s), 7.13-7.29 (7H, m), 7.41-7.49 (3H, m), 7.55-7.66 (4H, m)	IR (KBr): 3186, 3061, 1665, 1633, 1591, 1559 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{23}H_{19}N_4O$ ($[M+H]^+$) 367.1553 ; found 367.1567	13.2	97.8
22	NMR ($CDCl_3$,δ): 2.00 (3H, s), 2.44 (3H, br s), 7.06 (1H, br s), 7.15-7.50 (7H, m), 8.43 (2H, br s)	IR (KBr): 3173, 3026, 2923, 1662, 1652, 1634, 1617, 1558, 1508, 1473 cm^{-1}	ESI-HRMS (negative ion, sodium formate) calcd for $C_{18}H_{15}N_4O$ ($[M-H]^-$) 303.1246 ; found 303.1269	10.7	97.1
23	NMR (DMSO-d_6, δ): 7.14 (1H, t, *J* = 7.3 Hz), 7.20-7.30 (3H, m), 7.33 (2H, d, *J* = 7.3 Hz), 7.37-7.45 (3H, m), 7.45-7.55 (2H, m), 7.58 (1H, d, *J* = 8.7 Hz), 7.62 (1H, br s)	IR (KBr): 3101, 3073, 3059, 1651, 1596, 1572, 1555, 1510, 1466 cm^{-1}	ESI-HRMS (negative ion, sodium formate) calcd for $C_{22}H_{14}ClN_4O$ ($[M-H]^-$) 385.0856 ; found 385.0870	15.0	99.6
24	NMR ($CDCl_3$,δ): 2.39 (3H, s), 7.04 (2H, d, *J* = 7.8Hz), 7.05-7.50 (12H, m)	IR (KBr): 3419, 3059, 2974, 1641, 1615, 1600, 1565, 1513, 1469 cm^{-1}	ESI-HRMS (negative ion, sodium formate) calcd for $C_{23}H_{17}N_4O$ ($[M-H]^-$) 365.1402 ; found 365.1416	12.9	97.5
25	NMR (DMSO-d_6,δ): 2.16 (3H, s), 3.69 (2H, s), 7.13-7.19 (2H, m), 7.20-7.29 (2H, m), 7.32-7.36 (1H, m), 7.41-7.44 (1H, m), 7.50-7.55 (2H, m)	IR (KBr): 3280, 2900, 1666, 1619, 1573 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{18}H_{16}ClN_4O$ ($[M+H]^+$) 339.1007 ; found 339.1022	11.8	98.5
26	NMR (DMSO-d_6, δ): 2.19 (3H, s), 3.61 (2H, s), 7.13-7.19 (2H, m), 7.21-7.37 (4H, m), 7.50-7.55 (2H, m)	IR (KBr): 3350, 3068, 2919, 1629, 1553 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{18}H_{16}ClN_4O$ ($[M+H]^+$) 339.1007 ; found 339.1006	11.8	97.9
27	NMR (DMSO-d_6, δ): 2.17 (3H, s), 3.59 (2H, s), 7.13-7.19 (2H, m), 7.28-7.35 (4H, m), 7.49-7.55 (2H, m)	IR (KBr): 3259, 3027, 2935, 2909, 1556 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{18}H_{16}ClN_4O$ ($[M+H]^+$) 339.1007 ; found 339.1010	11.9	98.4
28	NMR (DMSO-d_6, δ): 2.16 (3H, s), 3.66 (2H, s), 7.14-7.19 (2H, m), 7.34-7.37 (2H, m), 7.50-7.55 (2H, m), 7.57-7.59 (1H, m)	IR (KBr): 3208, 3067, 2916, 2897, 1628, 1552 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{18}H_{15}Cl_2N_4O$ ($[M+H]^+$) 373.0617 ; found 373.0630	12.9	98.1
29	NMR (DMSO-d_6, δ): 2.19 (3H, s), 3.61 (2H, s), 7.14-7.19 (2H, m), 7.28 (1H, dd, *J* = 1.8 and 8.2Hz), 7.50-7.57 (4H, m)	IR (KBr): 3183, 2890, 1626, 1606 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{18}H_{15}Cl_2N_4O$ ($[M+H]^+$) 373.0617 ; found 373.0621	12.6	99.1
30	NMR (DMSO-d_6, δ): 2.18 (3H, s), 3.69 (2H, s), 7.12-7.18 (2H, m), 7.47-7.54 (4H, m), 7.63 (2H, d, *J*=8.2Hz)	IR (KBr): 3263, 2917, 1667, 1620, 1548 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{16}F_3N_4O$ ($[M+H]^+$) 373.1270 ; found 373.1287	12.2	98.9
31	NMR (DMSO-d_6, δ): 2.17 (3H, s), 3.59 (2H, s), 7.06-7.11 (2H, m), 7.14-7.18 (2H, m), 7.29-7.33 (2H, m), 7.51-7.54 (2H, m)	IR (KBr): 3246, 3069, 2910, 1657, 1605, 1507 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{18}H_{15}FN_4ONa$ ($[M+Na]^+$) 345.1122 ; found 345.1114	11.2	97.5
32	NMR (DMSO-d_6, δ): 2.15 (3H, s), 3.53 (2H, s), 3.70 (3H, s), 6.80-6.86 (2H, m), 7.13-7.21 (4H, m), 7.50-7.55 (2H, m)	IR (KBr): 3259, 3040, 2903, 2834, 1627, 1547 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{19}N_4O_2$ ($[M+H]^+$) 335.1503 ; found 335.1493	10.8	98.2
33	NMR (DMSO-d_6, δ): 2.20 (3H, s), 3.64 (2H, s), 7.12-7.19 (2H, m), 7.30-7.40 (3H, m), 7.41-7.47 (2H, m), 7.50-7.64 (6H, m)	IR (KBr): 3267, 3028, 1657, 1555 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{24}H_{21}N_4O$ ($[M+H]^+$) 381.1710 ; found 381.1712	12.6	98.5
35	NMR (DMSO-d_6,δ): 2.15 (3H, s), 2.39 (3H, s), 3.59 (2H, s), 6.96-7.00 (1H, m), 7.13-7.20 (1H, m), 7.23-7.29 (4H, m), 7.31 (1H, br s), 7.39 (1H, d, *J* = 8.2Hz)	IR (KBr): 3312, 3024, 2936, 2915, 1653, 1553 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{19}N_4O$ ($[M+H]^+$) 319.1559 ; found 319.1588	11.2	99.4

36	NMR (DMSO-d_6,δ): 2.17 (3H,s), 3.59 (2H,s), 7.13-7.21(2H,m), 7.24-7.30 (4H,m), 7.52 (1H, d,*J* = 8.7Hz), 7.55 (1H, d,*J* = 2.3Hz)	IR (KBr): 3263, 3031, 2914, 2842, 1654, 1623, 1556 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{18}H_{16}ClN_4O$ ([M+H]$^+$) 339.1007 ; found 339.0978	13.1	98.9
37	NMR (DMSO-d_6, δ): 2.17 (3H, s), 3.61 (2H, s), 7.13-7.22 (1H, m), 7.23-7.30 (4H, m), 7.32 (1H, dd, *J* = 8.2 and 1.8 Hz), 7.49 (1H, d, *J* = 8.2 Hz), 7.70 (1H, d, *J* = 1.8 Hz)	IR (KBr): 3030, 2923, 2850, 1706, 1637, 1592, 1576, 1545, 1509 cm^{-1}	ESI-HRMS (negative ion, sodium formate) calcd for $C_{18}H_{14}BrN_4O$ ([M-H]$^-$) 381.0345; found 381.0348	12.6	97.6
38	NMR (DMSO-d_6,δ): 2.19 (3H ,s), 3.60 (2H, s), 7.14-7.21 (1H, m), 7.24-7.31 (4H, m), 7.46-7.52 (1H,m), 7.70 (1H, d, *J* = 8.2 Hz), 7.84 (1H, s)	IR (KBr): 3033, 2935, 2901, 1637, 1551 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{16}F_3N_4O$ ([M+H]$^+$) 373.1271 ; found 373.1259	13.6	98.8
39	NMR (DMSO-d_6,δ): 1.33 (9H, s), 2.15 (3H, s), 3.59 (2H, s), 7.13-7.20 (1H, m), 7.22-7.30 (5H, m), 7.43 (1H, d, *J* = 8.2 Hz), 7.51 (1H, br s)	IR (KBr): 3026, 2961, 2903, 1655, 1558 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{22}H_{25}N_4O$ ([M+H]$^+$) 361.2023 ; found 361.2029	12.5	97.9
40	NMR (DMSO-d_6, δ): 2.18 (3H, s), 3.61 (2H, s), 7.14-7.20 (1H, m), 7.24-7.36 (5H, m), 7.43-7.50 (3H, m), 7.59 (1H, d, *J* = 8.2Hz), 7.64-7.68 (2H, m), 7.76 (1H, br s)	IR (KBr): 3338, 3025, 2897, 1623, 1577, 1541 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{24}H_{21}N_4O$ ([M+H]$^+$) 381.1710 ; found 381.1716	13.2	98.4
41	NMR (DMSO-d_6, δ): 2.18 (3H, s), 3.60 (2H, s), 7.14-7.20 (1H, m), 7.24-7.31 (4H, m), 7.46 (1H, dd, *J* = 1.4 and 8.2 Hz), 7.51 (2H, d, J = 8.4 Hz, d), 7.59 (1H, d, *J* = 8.2 Hz), 7.69 (2H, d, *J* = 8.4 Hz, d), 7.76 (1H, br s)	IR (KBr): 3308, 3028, 2915, 1655, 1555 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{24}H_{20}ClN_4O$ ([M+H]$^+$) 415.1302 ; found 415.1327	14.2	97.1
42	NMR (DMSO-d_6, δ): 2.17 (3H, s), 2.35 (3H, s), 3.60 (2H, s), 7.13-7.21 (1H, m), 7.23-7.31 (6H, m), 7.43 (1H, dd, *J* = 8.2 and 1.8 Hz), 7.51-7.59 (3H, m), 7.72 (1H, br s)	IR (KBr): 3446, 3027, 2962, 2873, 1632, 1556 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{25}H_{23}N_4O$ ([M+H]$^+$) 395.1866 ; found 395.1852	13.6	98.0
43	NMR (DMSO-d_6,δ): 2.18 (3H, s), 2.52 (3H, s), 3.61 (2H, s), 6.95-6.99 (1H, m), 7.05 (1H, t, *J* = 7.8 Hz), 7.13-7.21 (1H, m), 7.22-7.30 (4H, m), 7.35 (1H, d, *J* = 7.8 Hz)	IR (KBr): 3272, 3027, 1667, 1628, 1575 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{19}N_4O$ ([M+H]$^+$) 319.1559 ; found 319.1562	11.9	98.6
44	NMR (DMSO-d_6,δ): 2.17 (3H, s), 2.31 (3H, s), 2.44 (3H, s), 3.61 (2H, s), 6.96 (1H, d, *J* = 7.8 Hz), 7.12-7.30 (6H, m)	IR (KBr): 3026, 2920, 2866, 1677, 1600 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{20}H_{21}N_4O$ ([M+H]$^+$) 333.1710 ; found 333.1726	12.1	97.4
45	NMR (DMSO-d_6,δ): 2.17 (3H, s), 2.39 (3H, s), 3.69 (2H, s), 6.99 (1H, dd, *J*=0.9 and 8.2Hz), 7.32 (1H, br s), 7.39 (1H, d, *J* = 8.2 Hz), 7.50 (2H, d, *J* = 8.2 Hz), 7.64 (2H, d, *J* = 8.2 Hz)	IR (KBr): 3268, 2923, 2865, 1666, 1552 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{20}H_{18}F_3N_4O$ ([M+H]$^+$) 387.1433 ; found 387.1414	12.4	99.0
46	NMR (DMSO-d_6, δ): 2.14 (3H, s), 2.39 (3H, s), 3.51 (2H, s), 3.70 (3H, s), 6.81-6.85 (2H, m), 6.96-7.00 (1H, m), 7.16-7.21 (2H, m), 7.31 (1H, br s), 7.39 (1H, d, *J* = 8.2 Hz)	IR (KBr): 2921, 2833, 1673, 1651, 1583 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{20}H_{21}N_4O_2$ ([M+H]$^+$) 349.1659 ; found 349.1659	11.0	98.6
47	NMR (DMSO-d_6,δ): 2.17 (3H, s), 2.39 (3H, s), 3.60 (2H, s), 6.98 (1H, dd, *J*=0.9 and 8.2Hz), 7.27 (1H, dd, *J* = 1.8 and 8.2Hz), 7.32 (1H, br s), 7.39 (1H, d, *J* = 8.2 Hz), 7.52 (1H, d, *J* = 8.2 Hz), 7.55 (1H, d, *J* = 1.8 Hz)	IR (KBr): 3050, 2922, 2865, 1665, 1561 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{17}Cl_2N_4O$ ([M+H]$^+$) 387.0779 ; found 387.0748	12.7	97.8
48	NMR (DMSO-d_6, δ): 2.14 (3H, s), 2.40 (3H, s), 3.68 (2H, s), 6.99 (1H, d, *J* = 8.2 Hz), 7.19-7.36 (4H, m), 7.36-7.45 (2H, m)	IR (KBr): 3191, 2924, 2893, 1674, 1626, 1604cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{18}ClN_4O$ ([M+H]$^+$) 353.1163 ; found 353.1175	12.0	98.8
49	NMR (DMSO-d_6,δ): 2.15 (3H, s), 2.39 (3H, s), 3.58 (2H, s), 6.98 (1H, dd, *J* = 0.9 and 8.2Hz), 7.27-7.35 (5H, m), 7.39 (1H, d, *J* = 8.2Hz)	IR (KBr): 3032, 2921, 2864, 1665, 1552 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{18}ClN_4O$ ([M+H]$^+$) 353.1164 ; found 353.1151	12.0	98.1
50	NMR (DMSO-d_6, δ): 2.16 (3H, s), 2.39 (3H, s), 3.58 (2H, s), 6.98 (1H, dd, *J* = 0.9 and 8.2 Hz), 7.04-7.12 (2H, m), 7.26-7.34 (3H, m), 7.39 (1H, d, *J* = 8.2Hz)	IR (KBr): 3177, 3040, 2920, 1667, 1601 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{19}H_{18}FN_4O$ ([M+H]$^+$) 337.1459 ; found 337.1443	11.4	98.3
51	NMR (DMSO-d_6,δ): 1.24 (9H, s), 2.16 (3H, s), 2.39 (3H, s), 3.54 (2H, s), 6.98 (1H, dd, *J* = 0.9 and 8.2Hz), 7.17-7.21 (2H, m), 7.25-7.29 (2H, m), 7.31 (1H, br s), 7.39 (1H, d, *J* = 8.2 Hz)	IR (KBr): 3233, 3024, 2961, 2865, 1658, 1558 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{23}H_{27}N_4O$ ([M+H]$^+$) 375.2179 ; found 375.2184	13.1	98.4

52	NMR (DMSO-d_6,δ): 2.19 (3H, s), 2.39 (3H, s), 3.63 (2H, s), 6.96-7.00 (1H, m), 7.30-7.47 (7H, m), 7.54-7.64 (4H, m)	IR (KBr): 3246, 3031, 2922, 2864, 1656, 1557, 1541 cm^{-1}	ESI-HRMS (positive ion, sodium formate) calcd for $C_{25}H_{23}N_4O$ ($[M+H]^+$) 395.1866 ; found 395.1834	12.7	98.3

*) HPLC conditions
HPLC system (Tosho Co., Ltd.)
Autoinjector : AS-8020
Couln oven : CO-8020
liquid mixer : CO-8020
Pump : SD-8022 * 2
UV detector : UV-8020
Column: YMC-Pack Pro C18 (150 x 4.6 mm ID, S-5 um, 12 nm)

Gradient program

0 3 13 15 15.1 20 min
10 10 95 95 10 stop %acetonitrile in 0.1% TFA aq.
flow: 1 ml/min
detection: 254 nm