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Novel Metabolically Stable PCA-1/ALKBH3 Inhibitor Has Potent Antiproliferative Effects on DU145 Cells *In Vivo*

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Abstract. *Novel potent prostate cancer antigen-1 (PCA-1)/alpha-ketoglutarate-dependent dioxygenase alkB homolog 3 (ALKBH3) inhibitors both in vivo and in vivo were designed and evaluated by a stability assay in an S9 mixture, a mixture of rat liver homogenate and co-factors, and oral absorbability assay in rat, as well as enzyme and cell assays, and resulted in the synthesis of a novel potent PCA-1/ALKBH3 inhibitor in vivo. Among them, compound 7l exhibited potent inhibitory activities in a xenograft model bearing DU145 tumor at 10 mg/kg by subcutaneous administration without negative side-effects. This inhibitory activity in vivo was more potent than that of HUHS015 at 32 mg/kg, a known PCA-1/ALKBH3 inhibitor, or docetaxel at 2.5 mg/kg, the drug clinically used for androgen-independent prostate cancer.*

Developing novel drugs against prostate cancer that are effective against both androgen-dependent and independent cancer types is now urgently required (1). Tsujikawa *et al.* reported a novel gene that encodes a DNA and RNA-alkylating damage-repair enzyme called prostate cancer antigen (PCA)-1/alpha-ketoglutarate-dependent dioxygenase alkB homolog 3 (ALKBH3) that is highly expressed in clinical prostate cancer cells; genetic inhibition by injecting siRNA *in vivo* effectively inhibited the growth of androgen-independent prostate cancer cells DU145 cells that also express high levels of PCA-1/ALKBH3 (2-4). Suppression of PCA-1/ALKBH3 led to 3-methylcytosine accumulation and reduced cell proliferation in various cell lines (5). Therefore, a small and orally available PCA-1/ALKBH3 inhibitor could be a novel and clinically effective anti-prostate cancer drug, even for hormone-independent cancer. In previous work, we reported the first PCA-1/ALKBH3 inhibitor, HUHS015 (Figure 1), and its effectiveness in a xenograft model bearing DU145 tumors without any observable side-effects or toxicity. However, the inhibitory activity of HUHS015 was not sufficient especially after 1 week, although subcutaneous injection of 32 mg/kg HUHS015 completely suppressed the growth of DU145 cells in xenograft model up to 1 week (Figure 2) (6). Interestingly, in continuous study using rat S9 mixture, a mixture of rat liver homogenate including many metabolic enzymes and co-factors that is often used to estimate a compound's metabolic stability, we found that no HUHS015 was not detected in a S9 mixture at 37˚C after a 10-min treatment and only 42% of HUHS015 remained even at 15˚C indicating that HUHS015 was easily decomposed in liver *in vivo* after administration. In fact, only 0.08 μg/ml of HUHS015 was found in rat serum 60 min after oral administration of 32 mg/kg, while no metabolite so far has been identified, and its suppressory effects *in vivo* were not adequate in a xenograft model after long-term administration (Figure 2). Accordingly, we next focused our efforts to obtain novel PCA-1/ALKBH3 inhibitors that were more stable and effective in *in vivo* assays during the administration period. We herein report the design of novel PCA-1/ALKBH3 inhibitor and *in vitro* as well as *in vivo* structure–activity relationship studies, stability assay in S9 mixture, oral absorbability assays in rat, as well as enzyme and cell assays.

Materials and Methods

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's 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.

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Key Words: Anti-prostate cancer drug, docetaxel, DU145, xenograft model, anticancer drug, metabolic reaction, hepatic microsome, S9 mixture, drug design.

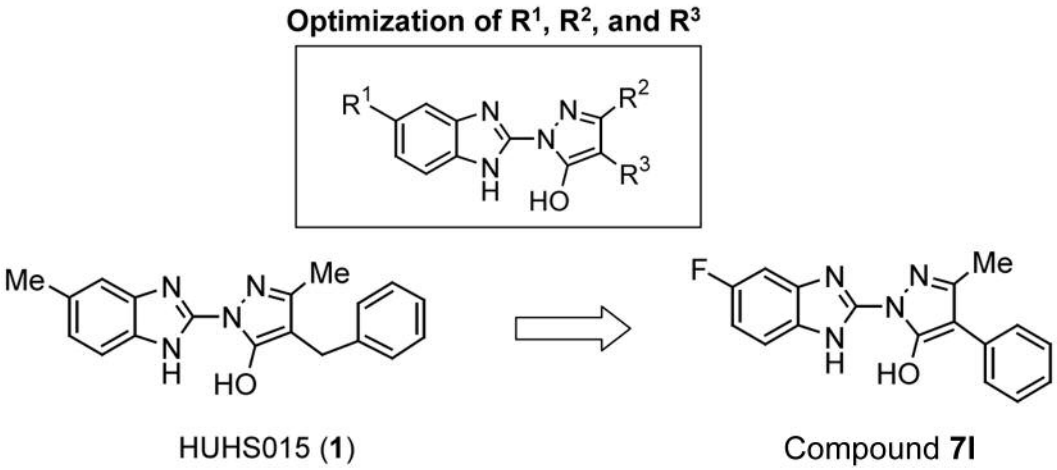

Figure 1. *Structure of inhibitors studied.*

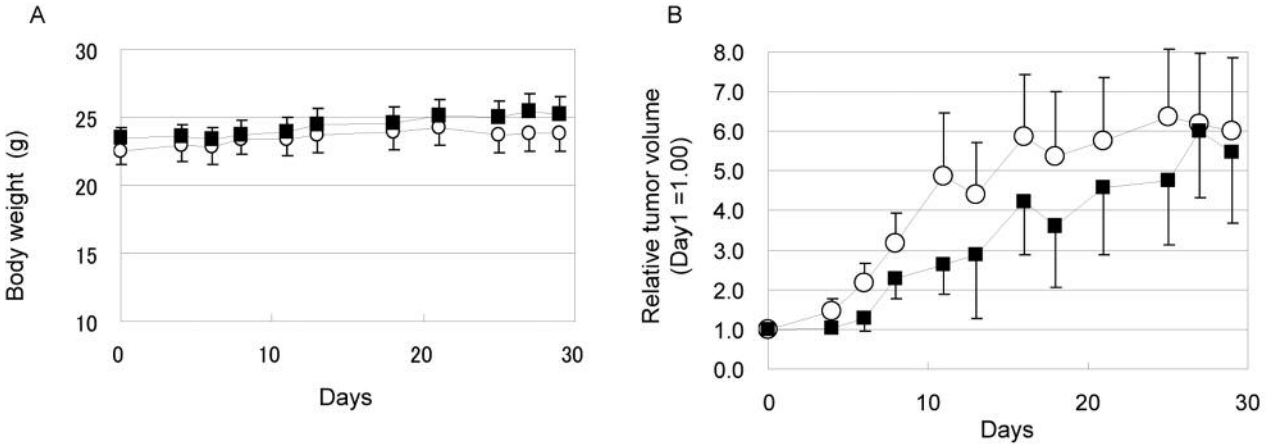

Figure 2. *The body weights and anti-proliferative effects of HUHS015 in mice bearing DU145 cell xenografts. Male nude mice were implanted subcutaneously with approximately 8×10⁶ DU145 cells mixed with Matrigel™ into the right flank. When the estimated tumor volume had reached almost 200 mm³, after randomization, mice were treated with HUHS015 subcutaneously administered once a day at 32 mg/kg (open symbols) or with vehicle alone (closed symbols). A: Body weights were measured at intervals. B: Tumor volume was calculated by caliper measurements of the width (W) and length (L) (volume=L×W²/2), and tumor volumes were estimated relative to those at day 0. Each value represents the mean±SE of six mice. No statistically significant differences were observed by Student's t-test.*

Animal care. Male nude BALB/c mice and male Sprague-Dawley (SD) rats were purchased from Japan SLC Inc. (Shizuoka, Japan) at 4 or 6 weeks old, almost 13 or 200 g body weight. 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 (#2013-01, 2013-19, 2015-28, and 2015-29).

Synthesis of compounds studied in this study. The substituted pyrazoles studied in this work were synthesized by condensing substituted hydrazines (**5**) with β-keto esters (**6**) as shown in Figure 3. Hydrazine derivatives (**5**) were synthesized from o-phenylenediamines (**2**) *via* 1,3-dihydro-benzimidazol-2-ones (**3**) and 2-chloro-benzimidazoles (**4**). ^{1}H-NMR spectra were recorded on a JEOL JNM-ECX400 spectrometer at 400 MHz, or Agilent NMR system PS 600 system at 600 MHz using $CDCl_3$, dimethylsulfoxide (DMSO)-d_6, CD_3OD or acetone-d_6 as solvent. Chemical shifts were given in δ values (ppm), using tetramethylsilane (δ=0.00) as the internal standard; coupling constants (*J*) were given in Hz. Signal multiplicities were characterized as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad signal). IR spectra were recorded on a JEOL FT-IR 4100 spectrometer. ESI-MS spectra were taken on Bruker microTOF-Q mass spectrometer. All reactions were monitored by thin-layer chromatography carried out on a 0.25 mm E. Merck silica gel plates 60 F_{254} using UV light, iodine, or 5% ethanolic phosphomolybdic acid solution and heat as developing agents.

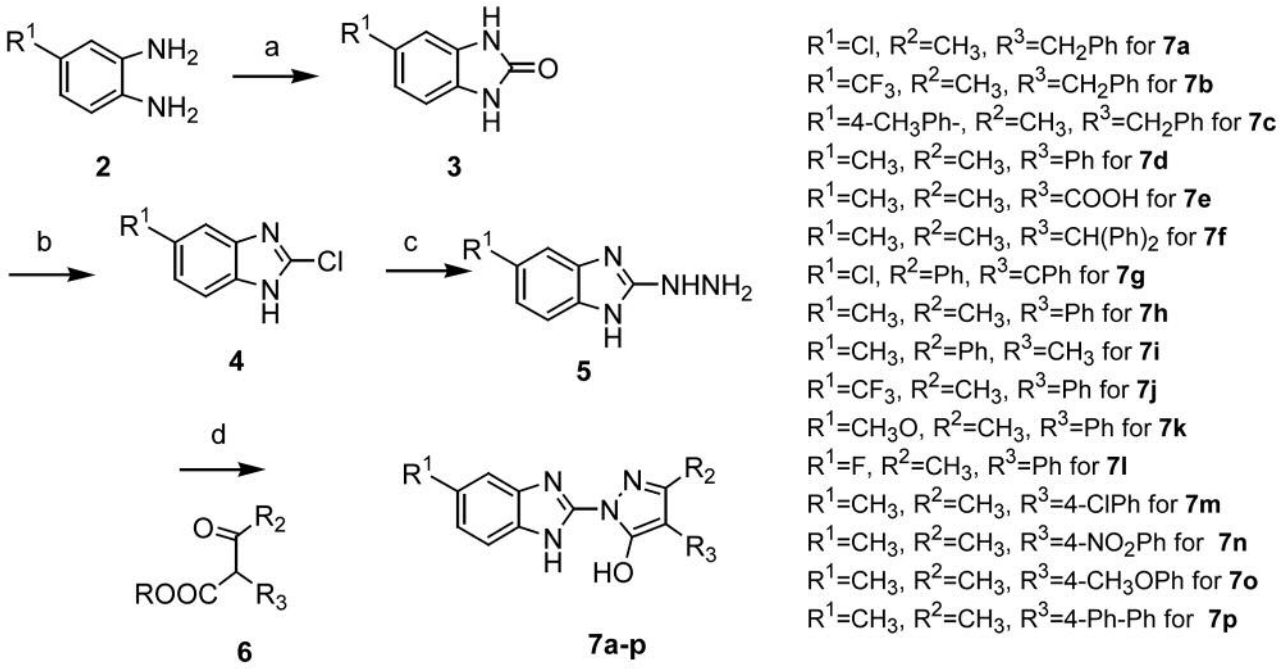

Figure 3. *Scheme for synthesis of compounds studied in this work. Reagents and conditions: (a) 1,1-carbonyldiimidazole, (b) $POCl_3$, (c) NH_2NH_2 hydrate, (d)* ***6****, in AcOH.*

*Synthesis of 5-fluoro-1H-benzo[d]imidazol-2(3H)-one (**3l**).* To a solution of 4-fluorobenzene-1, 2-diamine (**2l**, R^1=F, 5.20 g, 41.2 mmol) in THF (50 ml) were added 1,1-carbonyldiimidazole (7.35 g, 45.4 mmol) in 5 parts under ice-cooling. After being stirred for 2 h at room temperature, the reaction mixture was added to 1N HCl under ice-cooling. The reaction mixture was filtered and dried under reduced pressure to give 5-fluoro-*1H*-benzo[d]imidazol-2(*3H*)-one (**3l**, R^1=F, 5.20 g), was used without further purification. 1H-NMR (400MHz, DMSO-d_6) δ 6.73 (1H, ddd, J=10.4 (J_{HF}), 8.4 and 2.4 Hz), 6.76 (1H, dd, J=9.2 (J_{HF}), and 2.4 Hz), 6.87 (1H, dd, J=8.4 and 4.8 (J_{HF}) Hz), 10.6 (1H, s), 10.7 (1H, s).

*Synthesis of 5-fluoro-2-hydrazino-1H-benzo[d]imidazole (**5l**).* A mixture of 5-fluoro-1H-benzo[d]imidazol-2(*3H*)-one (**3l**, R^1=F, 5.20 g) and phosphoryl chloride (32 ml, 341 mmol) was stirred for 24 h at 120˚C. After cooling to ambient temperature, the mixture was added to 1N NaOH (resulting pH was almost 8). The aqueous layer was extracted with ethyl acetate for three times. The combined organic layers were washed with water and brine, then dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (*n*-hexane/ethyl acetate=1/1) to give compound **4l** (R^1=F, 4.22 g, 60% by 2 steps) as pale yellow precipitates. (400MHz, DMSO-d_6) δ 7.09 (1H, ddd, *J*=9.2 (J_{HF}), 8.8 and 2.8 Hz), 7.36 (1H, dd, *J*=9.2 (J_{HF}), and 2.8 Hz), 7.52 (1H, dd, *J*=8.8 and 4.8 (J_{HF}) Hz); IR (KBr): 3035, 2959, 2900. 2784, 2636, 1440. 1348, 1148, 1108 cm^{-1}; ESI-HRMS (positive ion, sodium formate): calcd for $C_7H_3ClFN_2$ ([M-H]$^-$) 168.9974; found 168.9986; m.p.146-149˚C. A mixture of **4l** (1.30 g, 7.62 mmol) and hydrazine monohydrate (7.30 ml, 152 mmol) was stirred for 2.5 h at 120˚C. After being cooled to ambient temperature, the reaction mixture was added to water. NaCl was added to the mixture and stirred at ambient temperature, the resulting precipitates were collected by filtration. The precipitates were washed with water, and then dried *in vacuo* to give 5-fluoro-2-hydrazino-1H-benzo[d]imidazole (**5l**, R^1=F, 720 mg, 56%) as white solid. ^{1}H-NMR (400MHz, DMSO-d_6) δ 4.46 (2H, br s), 6.63 (1H, broad), 6.87 (1H, dd, *J*=10.0 (J_{HF}) and 1.6 Hz), 7.05 (1H, dd, *J*=8.0 and 4.4 (J_{HF}) Hz), 7.89 (1H, broad), 11.0 (1H, broad): 3318, 3272, 3025, 2786, 1672, 1563, 1480. 1445, 1409, 1293, 1243, 1213, 1148, 1009 cm^{-1}; ESI-HRMS (positive ion, sodium formate): calcd for $C_7H_6FN_4$ ([M-H]$^-$) 165.0581; found 165.0597; m.p.189-193.

*Synthesis of 1-(5-fluoro-1H-benzimidazol-2-yl)-3-methyl-4-phenyl-1H- pyrazol-5-ol (**7l**).* A mixture of **5l** (700 mg, 4.21 mmol) and ethyl 3-oxo-2-phenylbutanoate (**6l**, 928 mg, 4.21 mmol) in acetic acid (7 ml) was stirred for 12 h at ambient temperature. The reaction mixture was poured into water (30 ml) and stirred at ambient temperatures. The resulting precipitates were collected by filtration, and washed water and then with 50% EtOH to afford (**7l**, 880 mg, 68%). The other compounds studied in this work were prepared in a similar manner, and their analytical data are provided in Table I.

PCA-1-inhibitory activity. An assay system for PCA-1 demethylase inhibitors has been described in full elsewhere (6). Briefly, 4 ng of PCA-1 with or without investigatory compounds was incubated with 80 fmol 3-methyl cytosine oligo DNA (100 b.p.) (GeneDesign, Inc., Ibaraki, Osaka, Japan) as a substrate to be demethylated in buffer [50 mM Tris-HCl (pH 8.0), 2 mM ascorbic acid, 100 μM oxoglutarate, and 40 μM ferrous sulfate] and incubated at 37˚C for 1 h. The reaction was stopped by dilution to 20 times volume by

Table I. *Analytical data of HUHS015 (**1**) and evaluated compounds (**7a-7p**).*

Compound	^{1}H-NMR, δ values*	IR (KBr)	ESI-HRMS	Melting point (˚C)
1	DMSO: 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)	3312, 3024, 2936, 2915, 1653, 1553 cm^{-1}	Calcd for $C_{19}H_{19}N_4O$ ([M+H]$^+$) 319.1559; found 319.1588	198-200
7a	DMSO: 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)	3263, 3031, 2914, 2842, 1654, 1623, 1556 cm^{-1}	Calcd for $C_{18}H_{16}ClN_4O$ ([M+H]$^+$) 339.1007; found 339.0978	101-104
7b	DMSO: 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*=8.2Hz), 7.84(1H,s)	3033, 2935, 2901, 1637, 1551 cm^{-1}	Calcd for $C_{19}H_{16}F_3N_4O$ ([M+H]$^+$) 373.1271; found 373.1259	216-218
7c	DMSO: 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*=1.8, 8.2Hz), 7.51-7.59 (3H, m), 7.72 (1H, br s)	3446, 3027, 2962, 2873, 1632, 1556 cm^{-1}	Calcd for $C_{25}H_{23}N_4O$ ([M+H]$^+$) 395.1866; found 395.1852	120-124
7d	DMSO: 2.39 (3H, s), 2.41 (3H, s), 7.07 (1H, dd, *J*=0.9 and 8.2Hz), 7.12-7.17 (1H, m), 7.32-7.38 (3H, m), 7.44 (1H, d, *J*=8.2Hz), 7.64-7.69 (2H, m)	3060. 1660. 1596, 1514 cm^{-1}	Calcd for $C_{18}H_{17}N_4O$ ([M+H]$^+$) 305.1397; found 305.1402	130-134
7e	DMSO: 2.14 (3H, s), 2.38 (3H, s), 3.21 (2H, s), 6.98 (1H, d, *J*=7.5 Hz), 7.31 (1H, s), 7.39 (1H, d, *J*=7.5 Hz)	3175, 3034, 1679, 1651, 1635, 1605, 1559, 1507 cm^{-1}	Calcd for $C_{14}H_{13}N_4O_3$ ([M-H]$^-$) 285.0993; found 285.0983	246-248
7f	DMSO: 2.08 (3H, s), 5.28 (1H, s), 7.13-7.24 (4H, m), 7.26-7.33 (8H, m), 7.48-7.53 (2H, m)	3229, 3025, 1656, 1559 cm^{-1}	Calcd for $C_{24}H_{21}N_4O$ ([M+H]$^+$) 381.1710; found 381.1707	165-167
7g	DMSO: 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)	3101, 3073, 3059, 1651, 1596, 1572, 1555, 1510. 1466 cm^{-1}	Calcd for $C_{22}H_{14}ClN_4O$ ([M-H]$^-$) 385.0856; found 385.0870	111-117
7h	CDCl3: 2.39 (3H, s), 7.04 (2H, d, *J*=7.8Hz), 7.05-7.50 (12H, m)	3419, 3059, 2974, 1641, 1615, 1600, 1565, 1513, 1469 cm^{-1}	Calcd for $C_{23}H_{17}N_4O$ ([M-H]$^-$) 365.1402; found 365.1416	Decomp.
7i	CDCl3: 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)	3173, 3026, 2923, 1662, 1652, 1634, 1617, 1558, 1508, 1473 cm^{-1}	Calcd for $C_{18}H_{15}N_4O$ ([M-H]$^-$) 303.1246; found 303.1269	118-123
7j	CDCl3: 2.37 (3H, s), 7.32 (1H, t, *J*=7.2Hz), 7.40-7.50 (2H, broad), 7.45 (H, t, *J*=7.2 Hz), 7.61 (2H, d, *J*=7.2 Hz), 7.60-7.78 (1H, broad).	3435, 2922, 2854, 1637, 1553, 1437, 1399, 1329, 1250, 1220, 1164, 1051 cm^{-1}	Calcd for $C_{18}H_{12}F_3N_4O$ ([M-H]$^-$) 357.0969; found 357.0977.	117-120
7k	CDCl3: 2.35 (3H, s), 3.66 (3H, s), 6.81 (1H, dd, *J*=9.0 and 2.4Hz), 6.80-6.93 (1H, broad), 7.15-7.30 (1H, broad), 7.26 (1H, t, *J*=7.2 Hz), 7.41 (2H, t, *J*=7.2 Hz), 7.67 (2H, d, *J*=7.2 Hz).	3464, 3055, 2948, 2832, 1656, 1597, 1551, 1512, 1435, 1401, 1367, 1317, 1278, 1197, 1157, 1073, 1027 cm^{-1}	Calcd for $C_{18}H_{16}N_4O_2$ ([M+H]$^+$) 321.1346; found 321.1336.	127-132
7l	DMSO: 2.40 (3H, s), 7.65 (1H, ddd, *J*=9.0 (H-F), 8.4 and 2.0 Hz), 7.21 (1H, t, *J*=8.4 Hz), 7.35 (1H, dd, *J*=9.0 (H-F) and 2.0 Hz), 7.40 (2H, t, *J*=8.4 Hz), 7.54 (1H, dd, *J*=8.4 and 4.8 (H-F) Hz), 7.65 (2H, d, *J*=8.4 Hz).	3463, 3066, 2922, 2857, 1665, 1556, 1512, 1491, 1432, 1374, 1307, 1272, 1247, 1137, 1105, 1007 cm^{-1}	Calcd for $C_{17}H_{13}FN_4ONa$ ([M+Na]$^+$) 331.0966; found 331.0968.	115-119
7m	DMSO: 2.38 (3H, s), 2.41 (3H, s), 7.10 (1H, dd, *J*=8.4 and 1.8 Hz), 7.36 (1H, d, *J*=1.8 Hz), 7.36 (2H, d, *J*=8.4 Hz), 7.45 (1H, d, *J*=8.4 Hz), 7.73 (2H, d, *J*=8.4 Hz).	3029, 2954, 2903, 1669, 1638, 1591, 1513, 1434, 1396, 1229, 1206, 1182, 1132, 1093 cm^{-1}	Calcd for $C_{18}H_{14}ClN_4O$ ([M-H]$^-$) 337.0862; found 337.0847.	>250 (decomp.)
7n	DMSO: 2.43 (3H, s), 2.48 (3H, s), 7.18 (1H, dd, *J*=8.4 and 0.9 Hz), 7.39 (1H, br s), 7.48 (1H, d, *J*=8.4 Hz), 8.06 (2H, d, *J*=9.6 Hz), 8.13 (2H, d, *J*=9.6 Hz).	2426, 1680. 1618, 1594, 1520, 1490, 1436, 1377, 1240, 1204, 1111, 1034 cm^{-1}	Calcd for $C_{18}H_{14}N_5O_3$ ([M-H]$^-$) 348.1102; found 348.1098.	143-147
7o	CDCl3: 2.30 (3H, s), 2.36 (3H, s), 3.83 (3H, s), 6.97 (1H, d, *J*=7.8 Hz), 6.99 (2H, d, *J*=7.8 Hz), 7.12 (1H, br s), 7.20 (1H, d, *J*=7.8 Hz), 7.58 (2H, d, *J*=7.8 Hz).	3046, 2998, 2948, 2907, 2834, 1655, 1522, 1440. 1374, 1245, 1132, 1091, 1022, 1004 cm^{-1}	Calcd for $C_{19}H_{19}N_4O_2$. ([M+H]$^+$)335.1502; found 335.1491	120-124
7p	CDCl3: 2.39 (3H, s), 2.41 (3H, s), 7.04 (1H, d, *J*=8.4Hz), 7.10-7.35 (2H, broad), 7.35 (1H, t, *J*=7.6 Hz), 7.45 (2H, dd, *J*=8.0 and 7.6 Hz), 7.63 (2H, d, *J*=8.0 Hz), 7.66 (2H, d, *J*=7.6 Hz), 7.76 (2H, d, *J*=7.6 Hz).	3027, 2952, 2910. 2867, 1738, 1659, 1529, 1490. 1431, 1303, 1239, 1031, 1007 cm^{-1}	Calcd for $C_{24}H_{19}N_4O$ ([M-H]$^-$) 379.1564; found 379.1572.	139-145

*DMSO: measured in DMSO-d_6, $CDCl_3$: measured in $CDCl_3$.

Table II. *Inhibition of prostate cancer antigen-1 (PCA-1) and DU145 cell growth, stability in S9 mixture after 10 min at 15˚C, and serum concentration at 60 min after oral administration of 32 mg/kg 2-(5-substituted benzimidazol-2-yl)-4,5-substituted-3-hydroxy-pyrazoles.*

Compound	Structure[a]			PCA-1 inhibition, IC_{50} (μM)	DU145 cell growth inhibition (%)		Remainder after reaction with S9 mixture	Concentration in serum (μg/ml)
	R^1	R^2	R^3		At 1 μM	At 10 μM		
1 (HUHS015)	CH_3	CH_3	CH2Ph	0.7	35	54	42%	0.08
7a	Cl	CH_3	CH_2Ph	4.5	24	36	81%	0.15
7b	CF_3	CH_3	CH_2Ph	5.9	34	47	72%	0.02
7c	4-CH_3-Ph-	CH_3	CH_2Ph	12.0	78	61	64%	ND[b]
7d	CH_3	CH_3	Ph	9.7	NT[c]	32	74%	2.45
7e	CH_3	CH_3	COOH	>10 (37% at 10 mM)	–20	–11	87%	0.22
7f	CH_3	CH_3	$CH(Ph)_2$	7.2	52	72	85%	0.17
7g	Cl	Ph	Ph	6.9	31	38	76%	1.03
7h	CH_3	Ph	Ph	>10 (49% at 10 mM)	12	19	98%	0.31
7i	CH_3	Ph	CH_3	>10 (6% at 10 mM)	-6	27	35%	0.33

[a]See Figure 3. [b]ND: Not detected. [c]NT: Not tested.

water. Two microliters of each sample produced was then used as template in real-time polymerase chain reaction (PCR) using Bio-Rad iQ SYBR Green Supermix to 20 μl. The cycling conditions consisted of an initial single cycle at 95˚C for 10 s followed by 40 cycles at 95˚C for 5 s, at 61˚C for 30 s, and at 72˚C for 15 s. The PCA-1-inhibitory effects of compounds were determined by quantitative analysis with real-time PCR.

Inhibitory effect of compounds on proliferation of DU145 cells. 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 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST) assay, which is determined by measuring the capacity of the cells to reduce WST to formazan. Briefly, 5×10^3 cells/well in 90 μl culture medium were incubated in the presence or absence of each compound for 48 h. 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 h. Absorbance was then measured using a microplate reader with a test wavelength of 450 nm and a reference wavelength of 630 nm. The percentage of inhibition by each compound was calculated, compared with 0.1% DMSO-treated control.

Measurement of compound stability in S9 mixture. The tested compound (2 mg/ml, reaction volume 1 ml) was added to a S9 mixture with co-factors (#S9MIX; IEDA Co., Ltd., Tokyo, Japan) for 10 min at 15˚C or 0˚C as control (7). After washing with n-hexane, the target compounds were twice extracted with ethyl acetate. After removal of solvents by a speed-vac apparatus (KOIKE Precision Instruments Co., Ltd., Itami, Japan), the amount of compound was then determined using an HPLC system (#8020; TOSOH Co., Ltd., Tokyo, Japan) with YMC-Pack Pro C18 (YMC Co., Ltd., Kyoto, Japan) at 1.0 ml/min of 0.1% TFA/H_2O as mobile phase A, 0.1% TFA/CH_3CN as mobile phase B. The amount of the compound remaining in the S9 mix was determined compared with the control.

Measurement of serum concentration of compounds in rats after oral administration. Male SD rats were fasted for 18 h then 10 or 32 mg/kg of compound was suspended in 0.5% methylcellulose (MC) and orally administered. At 30 or 60 min after administration, blood samples were obtained from abdominal vein or subclavian vein under anesthesia and centrifuged, the serum was separated and stored at –20˚C. The concentration of each compound in the serum was measured using HPLC as mentioned above.

Growth-inhibitory effect in mice bearing DU145 xenograft. Male nude mice were implanted subcutaneously with approximately 8×10^6 DU145 cells mixed with BD Matrigel™ Basement Membrane Matrix High Concentration (BD Biosciences Bedford, MA, USA) into the right flank. When the estimated tumor volume had reached almost 200 mm^3, after randomization into two treatment groups, HUHS015 or vehicle (50% DMSO, 15% EtOH, 35% sterile water) was subcutaneously administered once a day for 28 days.

Next, male nude mice were implanted subcutaneously with approximately 8×10^6 DU145 cells mixed with BD Matrigel™ Basement Membrane Matrix High Concentration into the right flank. When the estimated tumor volume had reached almost 200 mm^3, tumors were cut to ~10 mm^3 blocks and were then implanted subcutaneously into the right flank of another nude mouse of BALB/c background. When the estimated tumor volume in the mice had reached 100 to 300 mm^3, animals were divided into three experimental groups of six mice and treated subcutaneously with docetaxel (2.5 mg/kg, once a week) or compound **7l** (10 mg/kg, once a day) suspended in 0.5% MC for 25 days, all mice were equally administered 0.5% MC as vehicle. The tumor volume was calculated every 2 or 3 days using the following formula: tumor volume (mm^3)=$L\times W^2/2$, where L and W represent the length and the width of the tumor mass, respectively. The changes of tumor volume before administration were calculated. The day after last administration, blood samples were obtained from an abdominal vein under anesthesia and centrifuged, the serum was separated and stored at –20˚C. Serum concentrations of glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), creatinine, and blood urea nitrogen (BUN) were measured (WAKO

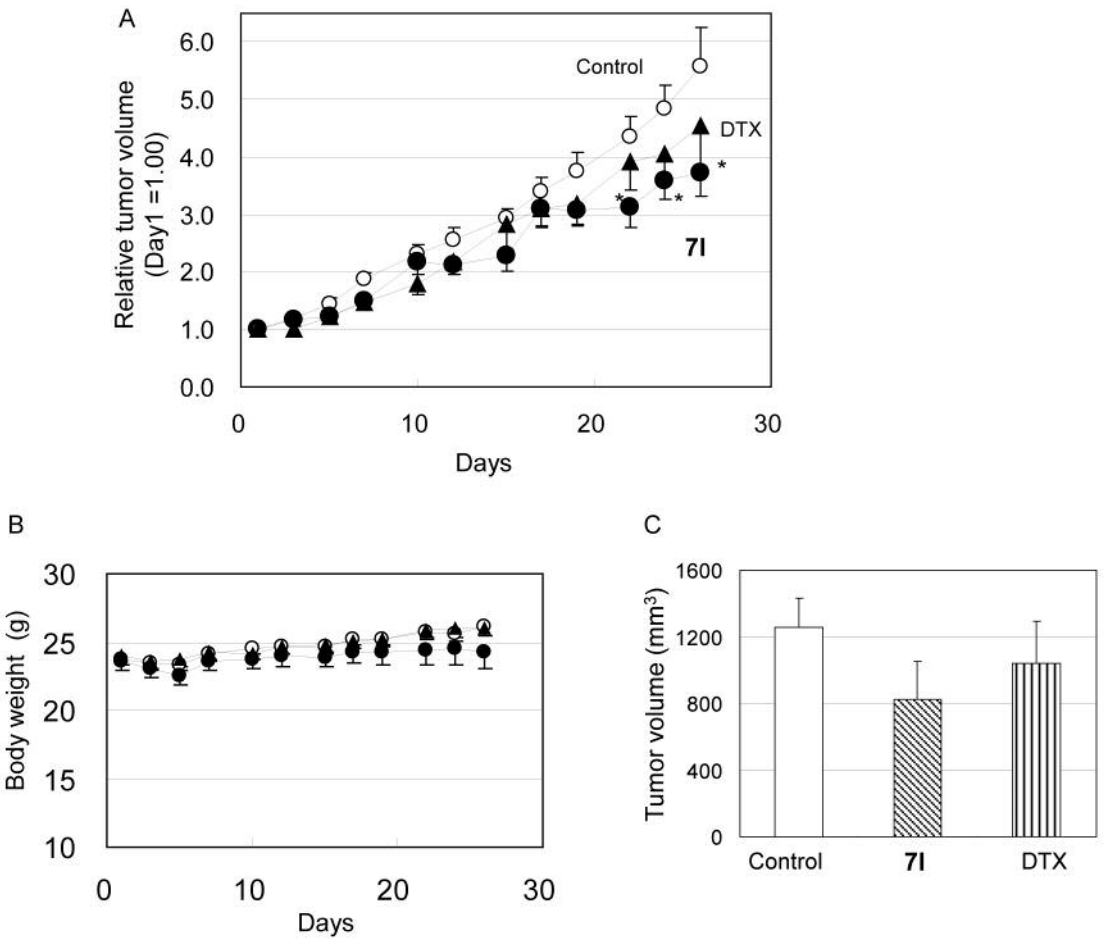

Figure 4. *The effects of compound **7l** [10 mg/kg, subcutaneously (s.c.), once a day] and docetacel (DTX, 2.5 mg/kg, s.c., once a week) on body weights and tumor in mice bearing DU145 cell xenografts. After randomization into three groups, mice were administered DTX, **7l**, or vehicle alone was administered s.c. once a week, or once a day, respectively. A: Tumor volume was calculated by caliper measurements of the width (W) and length (L) (volume=L×W2/2), and tumor volumes were estimated relative to those at day 1. B: Body weights were measured at intervals. C: Tumor volumes (mm3) were measured on the final day. Each value represents the mean±SE of six mice. *Significantly different at p<0.05 in comparison with the vehicle-treated control by Student's t-test.*

Table III. *Inhibition of prostate cancer antigen-1 (PCA-1) and DU145 cell growth, stability in S9 mixture after 10 min at 15˚C, and serum concentration after oral administration of 10 mg/kg 2-(5-substituted- benzimidazol-2-yl)-4-substituted-3-hydroxy-5- methyl-pyrazoles.*

Compound	Structure[a]			PCA-1 inhibition, IC_{50} (μM)	DU145 cell growth inhibition (%)		Concentration in serum (μg/ml) after oral administration	
	R^1	R^2	R^3		At 1 μM	At 10 μM	30 min	60 min
7j	CF_3	CH_3	Ph	1.6	60	78	1.00	0.33
7k	CH_3O	CH_3	Ph	1.0	65	78	0.63	0.18
7l	F	CH_3	Ph	2.9	63	77	1.02	0.73
7m	CH3	CH_3	4-ClPh	>10 (33% at 10 μM)	NT[b]		NT	
7n	CH3	CH_3	4-NO2Ph	>10 (39% at 10 μM)	NT		NT	
7o	CH3	CH_3	4-CH3OPh	>10 (18% at 10 μM)	NT		NT	
7p	CH3	CH_3	4-Ph-Ph	>10 (11% at 10 μM)	NT		NT	

[a]See Figure 3. [b]NT: not tested.

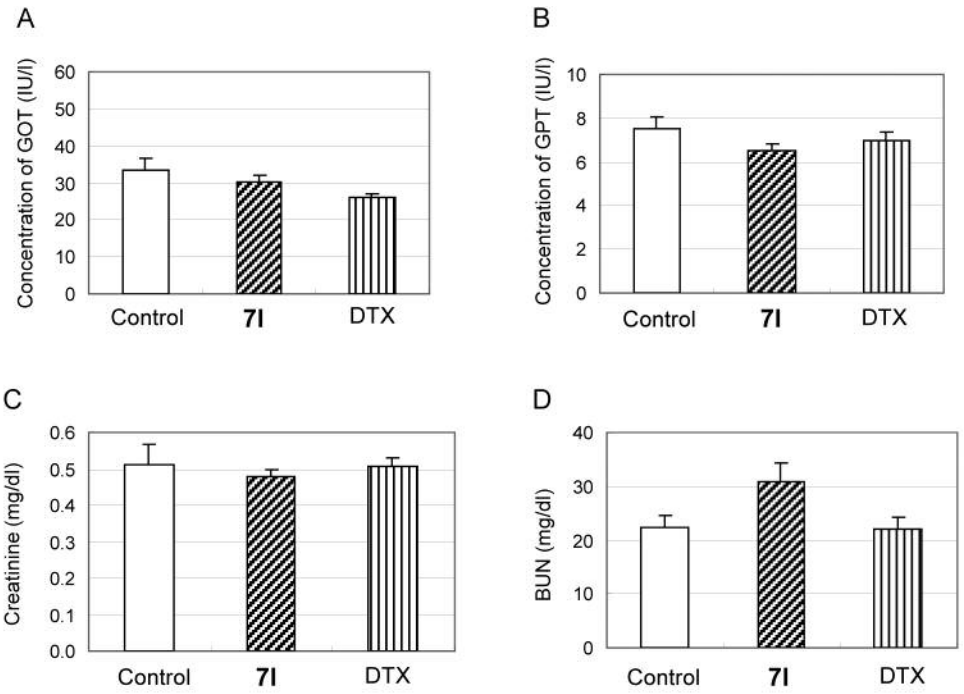

Figure 5. *Serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), creatinine, and blood urea nitrogen (BUN) of mice were measured on the final day. The values represent means±SE (n=6). No statistical significance was observed by Student's t-test.*

Pure Chemical Industries, Ltd. Chuo-ku, Osaka, Japan). Results are shown as means±SE of six mice and statistical significance of differences were analyzed by *t*-test.

Results and Discussion

We evaluated the stability of compounds studied in this work and estimated their anti-PCA-1/ALKBH3 and anti-DU145 cell growth activities *in vitro* (Tables II and III).

Replacement of the methyl at the 5-position of the benzimidazole ring (R1) with Cl (**7a**) produced almost 2-fold increase in stability in the S9 mixture treatment (**1**: 42% *vs*. **7a**: 81% remaining, Table II), and a higher serum concentration (0.15 μg/ml) compared to those of **1** (0.08 μg/ml), while anti-PCA-1/ALKBH3 activity was reduced [half maximal inhibitory concentration (IC_{50})=0.7 *vs*. 4.5 μM], indicating that the methyl was metabolized in the liver and that modifications which yielded derivatives with resistance to metabolic reactions in the S9 mixture would be effective for enhancement of *in vivo* activities.

Replacement of the methyl with trifluoromethyl (**7b**) and 4-Me-phenyl (**7c**) also afforded stable derivatives in the S9 mixture treatment (Table II), while anti-PCA-1/ALKBH3 and anti-DU145 growth activities were reduced. Compound **7c** was, curiously, not detected in serum, while it was relatively stable in the S9 mixture. We speculate that this result was due to the low solubility of **7c** (data not shown) and that **7c** might be precipitated in gastrointestinal tract after oral administration.

Replacement of the benzyl with a carboxylate (**7e**) resulted in good serum concentration (0.22 μg/ml, 60 min after oral administration), but showed only weak inhibitory activities on PCA-1/ALKBH3 and growth of DU145 cells. Derivatives bearing phenyl rings (**7d**, **7g-7h**) or a diphenylmethyl (**7f**) instead of a benzyl at the 4 position of the pyrazole were then synthesized because such benzyl moieties are often metabolized (Figure 1). Compounds **7d** and **7g** demonstrated good stability in the S9 mixture and good serum concentrations after oral administration (2.45 μg/ml and 1.03 μg/ml, respectively), which were more than 10 times higher than those of **1** (Table II). Based on these results, we fixed the phenyl at the 4 position of the pyrazole for further studies because such stability in the S9 mixture and good bioavailability properties were attractive.

Replacement of the methyl group of the benzimidazole in **7d**, which could be as easily metabolized by the liver as the methyl group of **1**, with putatively metabolically stable groups, trifluoromethyl (**7j**), methoxy (**7k**), and fluoro atom (**7l**), was carried out. Derivatives **7d**, **7j**, and **7l** exhibited serum concentrations of 1.00. 0.63, and 1.02 μg/ml at 30 min after oral administration at 10 mg/kg, respectively, in line with our expectations (Table III). Moreover, compounds **7k** and **7l**

exhibited more potent anti-growth activities compared to that of **1**, while their anti-enzymatic inhibitory activities were weaker than that of **1**. We thought this discordance between the enzyme assay and cell-based assay was due to differences in their membrane-permeability. Introductions of substitutions on the phenyl ring (**7m-7p**) produced disappointing inhibition of anti-PCA-1/ALKBH3 (Table III). Among this series, we selected compound **7l** for further studies because it exhibited the highest serum concentration both at 30 min and 60 min after oral administration (10 mg/kg) and adequate inhibitory activities both in enzymatic (IC_{50}=2.9 μM) and DU145 assays (63% inhibition at 1 μM). The serum concentration of **7l** was almost 10 times higher than that of HUHS015 (**1**) at 60 min after oral administration (10 mg/kg), and its anti-growth effects on DU145 were slightly more potent than those of 1, while its PCA-1/ALKBH3 inhibitory activity was weaker than that of **1**.

The growth inhibition by compound **7l** in the xenograft model bearing DU145 tumors was examined for 26 days (10 mg/kg, *s.c.*) and compared to the inhibition exerted by docetaxel at approximately its clinical dosage (8) (2.5 mg/kg, *s.c.*, Figure 4). Compound **7l** demonstrated potent growth-inhibitory activity compared to docetaxel without limiting weight gain, even after 26 days of continuous administration. The inhibitory activity of compound **7l** was more potent than that of **1**. Docetaxel administration at 2.5 mg/kg was a critical dose in the DU145-bearing xenograft model because continuous administration at this dose caused edema-like side-effects (9) that are often observed in clinical use. Serum concentrations of GOT, GPT, creatinine, and BUN were measured on the final day (Figure 5), and there were no obviously irregular values, indicating a low toxicity of compound **7l**.

Conclusion

We designed and synthesized metabolically stable HUHS015 derivatives and evaluated their stabilities in S9 mixture and their serum concentrations after oral administration because HUHS015 is easily metabolized and decomposed *in vivo*. We also estimated the anti-PCA-1 and anti-growth activities of these derivatives *in vitro* to obtain potent novel PCA-1/ALKBH3 inhibitors *in vivo*. As a result of modifications to **1**, replacements to two metabolically susceptive moieties, the methyl group at the 5 position of benzimidazole and the benzyl of **1**, to the more stable fluoro atom and phenyl group in compound **7l** were effective both in improving the stabilization in S9 mixture and in achieving a high serum concentration after oral administration. Compound **7l** exhibited potent inhibitory activities against DU145 tumors in a xenograft model without observable side-effects after subcutaneous administration for 26 days, as expected. Currently, further modifications of compound **7l** and other derivatives to obtain a clinical trial compound are in progress. Additionally, the effects of combination treatment with PCA-1/ALKBH3 inhibitors and prostate cancer drugs mainly used in clinic, such as docetaxel, are also in progress and will be reported in the near future.

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