



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



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ARTICLE INFO

Article history:

Received 20 November 2013

Revised 2 January 2014

Accepted 6 January 2014

Available online 11 January 2014

Keywords:

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.

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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)-1² 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}

The substituted pyrazoles evaluated in this work were synthesized by condensing *N*-substituted hydrazines (**3**) with ethyl acetate 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.

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

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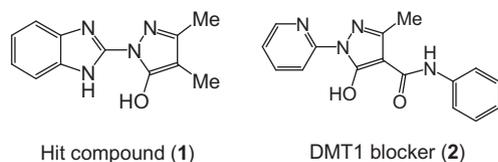
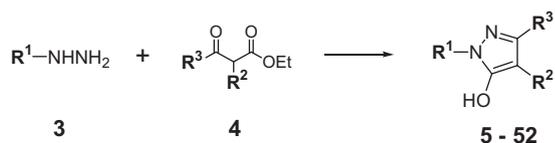


Figure 1. Structure of PCA-1/ALKBH3 inhibitor obtained in our random screening and previously known DMT1 blocker.



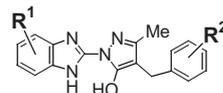
Scheme 1.

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*.

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

Table 2

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

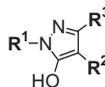


	R ¹	R ²	PCA-1 inhibition IC ₅₀ (μM)
25	H	2-Cl	>10 (–13%)
26	H	3-Cl	0.54
27	H	4-Cl	>10 (46%)
28	H	2,4-Cl ₂	>10 (11%)
29	H	3,4-Cl ₂	3.1
30	H	4-CF ₃	0.55
31	H	4-F	0.60
32	H	4-MeO	0.63
33	H	4-Phenyl	0.72
34	H	4-tBu	4.1
35	5-Me	H	0.67
36	5-Cl	H	4.5
37	5-Br	H	0.49
38	5-CF ₃	H	0.61
39	5-tBu	H	0.64
40	5-Phenyl	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%)
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

(**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

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	Benzimidazol-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-Naphthylmethyl	Me	0.75
17	Benzimidazol-2-yl	CH ₂ COOMe	Me	4.3
18	Benzimidazol-2-yl	CH ₂ COOH	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

^{*} PCA-1/ALKBH3 inhibition (%) at 10 mM are shown in parenthesis.

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 ($\mu\text{g/mL}$)
	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.

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.

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

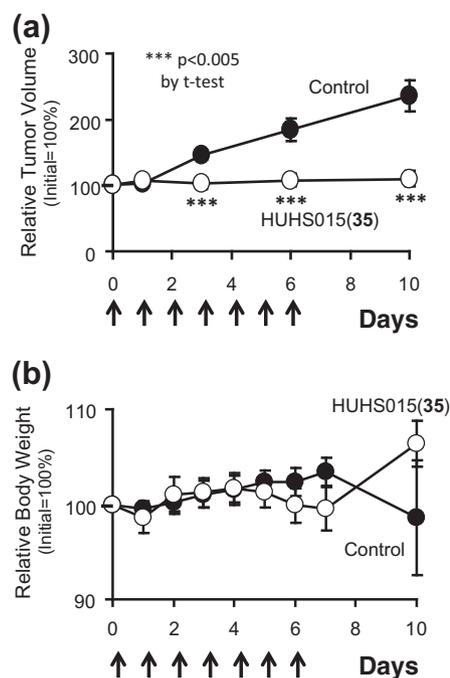


Figure 2. (a) Inhibitory effect of HUHS015 (**35**, 32 mg/kg/day, open circle) on the growth of subcutaneously implanted DU145 (nude mice) was examined ($n = 6$). Compound **35** or saline was administered for 6 days (arrows), and the tumor volume was measured for 10 days. Black circles demarcate the 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 \pm SE ($n = 6$).

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

This research was supported in part by a Grant-in-Aid for Scientific Research (C) provided by the Program for Promotion of Fundamental Sciences in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) and by a Grant-in-aid from the Knowledge Cluster Initiative (Second Stage) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2014.01.008>.

References and notes

- DeFrancesco, L. *Nat. Med.* **2001**, *7*, 1076.
- Konishi, N.; Nakamura, M.; Ishida, E.; Shimada, K.; Mitsui, E.; Yoshikawa, R.; Yamamoto, H.; Tsujikawa, K. *Clin. Cancer Res.* **2005**, *11*, 5090.
- Shimada, K.; Nakamura, M.; Ishida, E.; Higuchi, T.; Yamamoto, H.; Tsujikawa, K.; Konishi, N. *Cancer Sci.* **2008**, *99*, 39.
- Koike, K.; Ueda, Y.; Hase, H.; Kitae, K.; Fusamae, Y.; Masai, S.; Inagaki, T.; Saigo, Y.; Hirasawa, S.; Nakajima, K.; Ohshio, I.; Makino, Y.; Konishi, N.; Yamamoto, H.; Tsujikawa, K. *Curr. Cancer Drug Targets* **2012**, *12*, 847.
- Dango, S.; Mosammaparast, N.; Sowa, M. E.; Xiong, L. J.; Wu, F.; Park, K.; Rubin, M.; Gygi, S.; Harper, J. W.; Shi, Y. *Mol. Cell* **2011**, *44*, 373.
- Tasaki, M.; Shimada, K.; Kimura, H.; Tsujikawa, K.; Konishi, N. *Br. J. Cancer* **2011**, *104*, 700.
- Yamato, I.; Sho, M.; Shimada, K.; Hotta, K.; Ueda, Y.; Yasuda, S.; Shigi, N.; Konishi, N.; Tsujikawa, K.; Nakajima, Y. *Cancer Res.* **2012**, *72*, 4829.
- 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.
- Cadieux, J. A.; Zhang, Z.; Mattice, M.; Brownlie-Cutts, A.; Fu, J.; Ratkay, L. G.; Kwan, R.; Thompson, J.; Sanghara, J.; Zhong, J.; Goldberg, Y. P. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 90. 5-hydroxy-3-methyl-1-pyridin-2-yl-1H-pyrazole-4-carboxylic acid phenylamide, a representative compound in this work showed a $IC_{50} = 1.7 \mu M$ in PCA-1/ALKBH3 enzyme assay and 25% inhibition at $10 \mu M$ against proliferation of DU145 cells on dish..
- (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.
- No abnormal finding on mice's behavior and organs has been observed even after a 6-days continuous administration.
- Ringvoll, J.; Nordstrand, L. M.; Vågbo, C. B.; Talstad, V.; Reite, K.; Aas, P. A.; Lauritzen, K. H.; Liabakk, N. B.; Bjørk, A.; Dougherty, R. W.; Falnes, P. Ø.; Krokan, H. E.; Klungland, A. *EMBO J.* **2006**, *25*, 2189.