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Use of the Nitrile Oxide Cycloaddition (NOC) Reaction for Molecular Probe Generation: A New Class of Enzyme Selective Histone Deacetylase Inhibitors (HDACIs) Showing Picomolar Activity at HDAC6

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Abstract

A series of hydroxamate based HDAC inhibitors containing a phenylisoxazole as the CAP group has been synthesized using nitrile oxide cycloaddition chemistry. An HDAC6 selective inhibitor having a potency of ~2 picomolar was identified. Some of the compounds were examined for their ability to block pancreatic cancer cell growth and found to be about 10-fold more potent than SAHA. This research provides valuable, new molecular probes for use in exploring HDAC biology.

To date, considerable research activity has focused on understanding the “histone code” and, in particular, on the design of HDAC^a inhibitors as novel therapeutics for the treatment of a wide range of disorders, including cancer, as well as neurodegenerative diseases, and even malaria.¹ These compounds owe their action to their ability to reactivate silenced genes by modulating the condensation status of DNA. The post-translational acetylation status of chromatin is determined by the competing activities of two classes of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), which control the acetylation of lysine residues making up the histones. In general, HATs function to acetylate lysine groups in nuclear histones, resulting in neutralization of the charges on the histones and a more open, transcriptionally active chromatin structure, while the HDACs function to deacetylate and suppress transcription (the positively charged lysine amine group interacts with the negatively charge DNA organophosphate groups to cause compaction of the chromatin structure). A shift in the balance of acetylation on chromatin may result in changes in the regulation of patterns of gene expression.²

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Supporting Information **Available**: Experimental details and HPLC data of compounds **1–11**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

^aAbbreviations: Boc, *tert*-butyloxycarbonyl; CTCL, cutaneous T-cell lymphoma; DNA, deoxyribonucleic acid; EDCl, 1-ethyl-3-(3'-dimethyl-aminopropyl)carbodiimide; FDA, Food & Drug Administration; HATs, histone acetyltransferases; HDAC, histone deacetylase; HDACs, histone deacetylases; HDACIs, histone deacetylase inhibitors; HOBt, 1-hydroxy-benzotriazole; NOC, nitrile oxide cycloaddition; SAHA, suberoylanilide hydroxamic acid; TFA, trifluoroacetic acid; ZBG, zinc-binding group.

Because many cancers are associated with aberrant transcriptional activity and HDACs and HATs associate with both transcription activators and repressors, these enzymes have been identified as attractive targets for cancer therapy. Indeed, inhibitors of the HDACs have been shown to block tumor cell growth and induce differentiation and cell death.³ Several such inhibitory agents, including suberoylanilide hydroxamic acid (SAHA) and (*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]-4,21-di-isopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo[8.7.6]tricos-16-ene-3,6,9,19,22-pentone (FR901228)^{1b} have reached clinical trials, and SAHA has been approved by the FDA for use in cutaneous T-cell lymphoma (CTCL).⁴ HDACs work by allowing the transcription and expression of genes including tumor suppressor genes. HDAC inhibitors have also been shown to enhance the therapeutic efficacy of typical cytotoxic agents when tested in combination trials.⁵

While a fairly extensive array of HDACs has now been described, the majority of these fail to show any selectivity for the individual enzymes, at least as determined by using isolated enzyme assays. In most cases, such data are generally missing, as the assays were performed using a mixture of nuclear extracts. In our present efforts, and based upon other studies reported by us,⁶ we elected to investigate the HDAC selectivity of compounds readily assembled by use of nitrile oxide cycloaddition (NOC) chemistry (Figure 1), together with further studies of their action against pancreatic cancer cell lines. The present studies were thus designed to investigate issues of selectivity to arrive at molecular probes rather than to create candidate drugs per se. In this connection, it is now well recognized that the hydroxamate group represents a metabolic liability and may have some toxicity and that improved zinc binding groups will be needed to arrive at more druggable molecules. We have already reported some progress in this direction by employing a mercaptoacetamide group to bind to the catalytic zinc atom and shown these compounds to be superior in their neuroprotective ability in neuronal models of oxidative stress.^{6b}

The synthesis of compounds **1–11** was accomplished by making use of three related building blocks, namely the 5-arylisoxazole-3-carboxylic acid ethyl esters (**15–17**),⁷ which were synthesized according to the reported procedure (Scheme 1). Saponification of the ethyl ester followed by a standard peptide coupling reaction between the resulting acid and the variably sized long chain amino acid methyl ester using HOBt-EDCI provided the corresponding methyl ester. The ether-bearing analogue **19** was synthesized from alcohol **18** by reaction with methyl 5-bromovalerate in the presence of NaH. The alcohol **18** was prepared by NaBH₄ reduction of 5-phenylisoxazole-3-carboxylic ethyl ester **15**. Last, these methyl esters were converted to the corresponding hydroxamic acids by heating with hydroxylamine hydrochloride in the presence of base in accord with a literature procedure.⁸ For those compounds bearing an amino group, Boc deprotection was carried out using TFA prior to the formation of the hydroxamic acids.

All compounds were tested using the isolated enzymes under conditions described previously against both the class I (1, 2, 3, and 8) and class II (6 and 10) HDACs (Table 1 and Chart 1). Noteworthy is the observation that this set of structures show some selectivity for HDAC3 and 6, as they are less potent in the inhibition of HDAC1, -2, and -10, while showing only micromolar activity against HDAC8. Both the length of the linker chain and the amine-protecting group play a role in potency and selectivity. Thus compounds with a linker comprised of 6-methylene units and in their Boc-protected form (**3** and **7**) were highly potent and selective for HDAC3 and 6.

In fact, compound **7** shows an impressive potency of 2 picomoles against HDAC6 coupled with a superb level of enzyme selectivity. Given the importance of HDAC6 in the deacetylation of tubulin, the design of inhibitors against this target is of considerable interest.¹⁰ Replacement of the Boc group of **7** by acetyl as in **11** led to a considerable drop in

potency, suggesting the impact of this large lipophilic group on surface recognition. Compounds bearing an unsubstituted phenyl ring as in **1** and **2** show a similar trend in selectivity against HDAC3 and -6, although they are less potent, thus demonstrating that the phenylisoxazoles are readily amenable to structural modifications to refine their potency and selectivity.

Figure 2 shows inhibitor **7** docked in the binding site of our current homology model of HDAC6. The HDAC6 homology model was built using the X-ray crystal structure of HDAC7 (2PQP) as a template.¹¹ The ZBG (zinc-binding group) hydroxamic acid group and the six methylene groups bind deep inside the narrow binding pocket. The CAP region of the bound ligand appears to interact with only one side of the protein. The carbonyl group of the Boc group might interact with His499, which may be important in positioning the CAP residue on the surface of the HDAC protein. Such interactions are absent with the other inhibitors included in this study and may thus explain the higher potency observed for this compound. Preliminary investigations of other class I and class II HDAC homology models indicate that the loop areas surrounding the CAP residues show significant differences among the various HDACs and may eventually account for the enzyme selectivity that has been observed.

To further validate the utility of these HDACIs at the cellular level, we investigated their ability to block cancer cell growth. Because the ClogP values of these compounds are actually better than that of SAHA (Table 1), we believed that these ligands would be able to permeate the cell membrane. As pancreatic cancer is the fifth leading cause of cancer death in the U.S. and essentially remains an incurable disease with the incidence nearly equaling mortality, we chose these cell lines for study.¹² These cancers fail to respond to widely used chemotherapeutic drugs like 5-fluorouracil and gemcitabine,¹³ and thus there is a clear need for more effective drugs. The present HDACIs were tested against five transformed cell lines as well as against two normal cell lines. These data are provided in Table 2 along with comparison data using the now marketed drug SAHA. As is apparent, some of these isoxazoles show very good IC₅₀ values in the nanomolar range, and are more active than SAHA. Compounds **7** and **9**, for example, are active against both the Mia Paca-2 and Panc04.03 cell lines at the 100 nM level and at the 200–300 nM level against HupT3, whereas compound **5** is active against HupT3, Mia Paca-2 and SU86.86 at the 100–200 nM level. Of particular interest to the present study is the high activity shown by the relatively HDAC6 selective compound **7**, especially in view of its weaker HDAC1 activity, which is believed essential to the induction of apoptosis.¹⁴ Compounds **8**, **10**, and **11** which show poorer HDAC inhibition, show much weaker activity against these cell lines. Also, it should be noted that the activity of these isoxazoles against the transformed and nontransformed cell lines (HMEC and HPDE6c7) is no better than that shown by SAHA. Given the different levels of expression of the HDACs in the pancreatic cancer cells, the formation of multiprotein repressor complexes, as well as issues of distribution and metabolism, it is unlikely that one should expect a high correlation between the in vitro enzyme inhibitory activity and cellular cytotoxicity.

In summary, the present work demonstrates the ability of NOC chemistry to quickly generate a novel series of HDAC inhibitors, showing features of high potency and selectivity, as well as ability to inhibit pancreatic cancer cell growth. While human xenograft studies using these compounds are now underway, we anticipate that some of these compounds will become valuable molecular probes for further elucidating HDAC6 biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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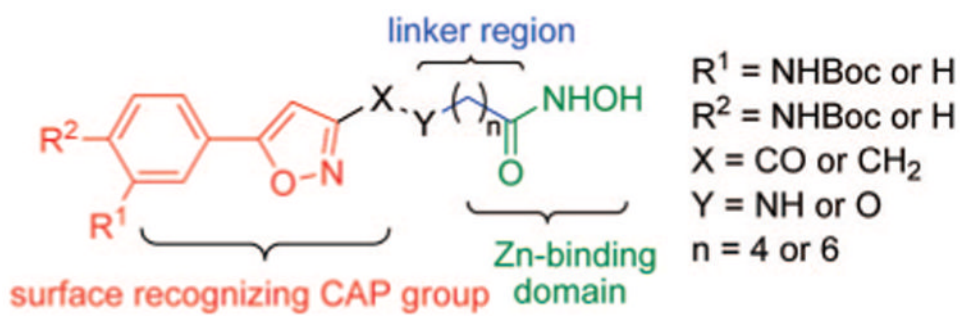


Figure 1.
General structural features of compounds 1–11.

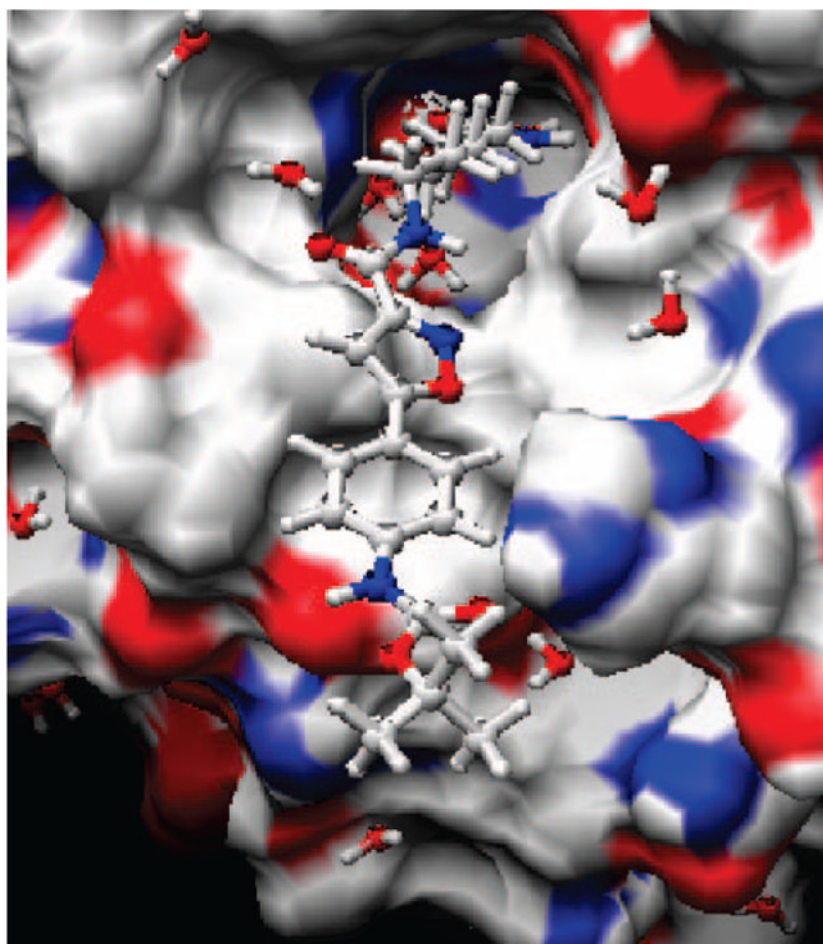


Figure 2.
Compound 7 docked into the binding site of the current HDAC6 homology model.

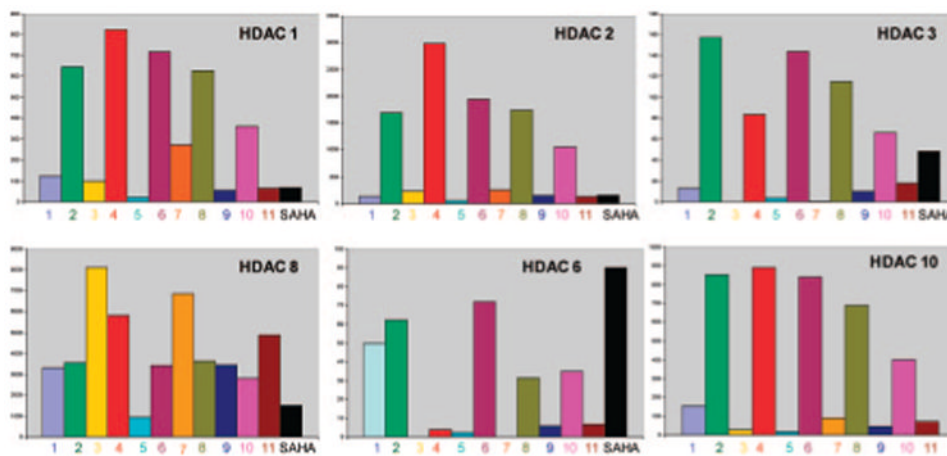
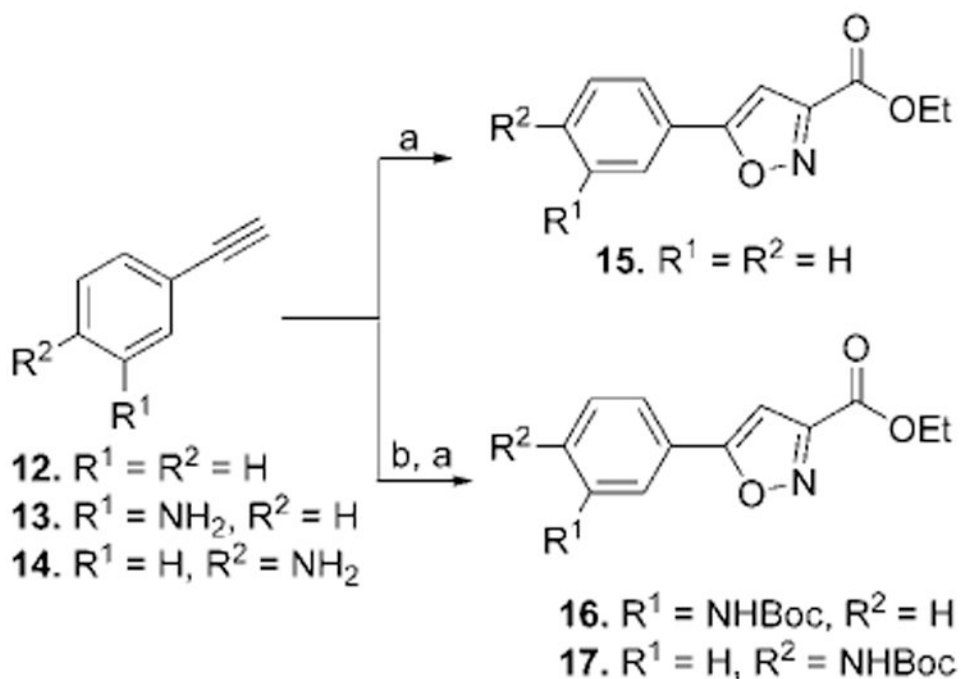
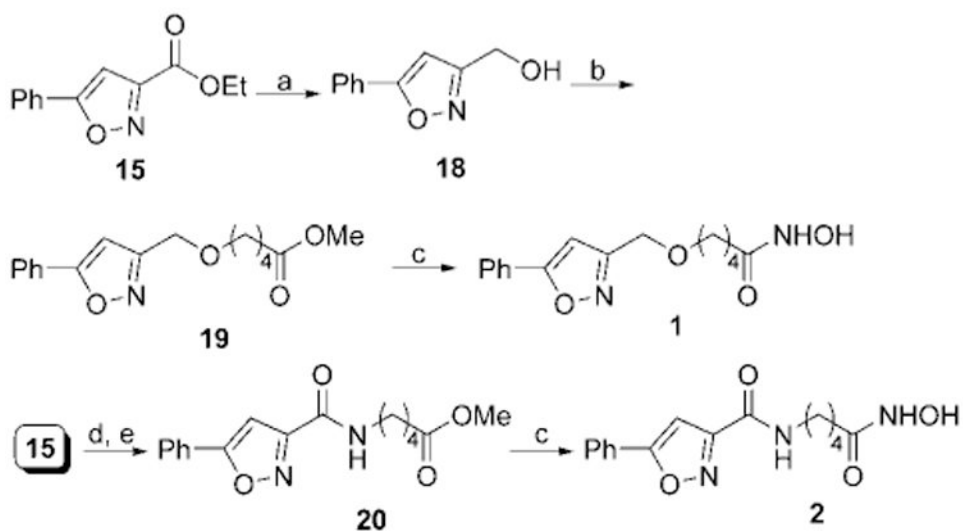


Chart 1. Comparison of IC₅₀ (nM) Values of Compounds 1–11 and SAHA against Class I and Class II HDACs



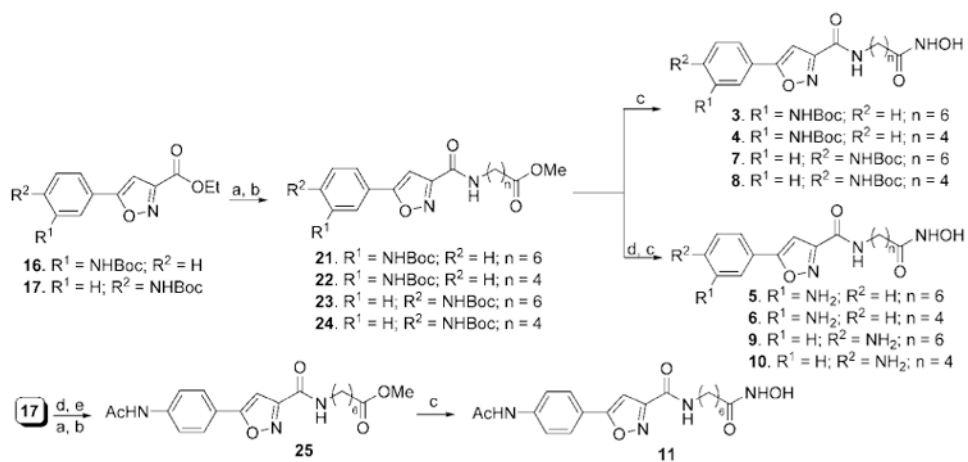
Scheme 1. Synthesis of Compounds 15–17^a

^a Reactions and conditions: (a) ethyl chlorooximidoacetate, triethylamine, THF, rt, 16 h; (b) (Boc)₂O, THF, reflux, 16 h.



Scheme 2. Synthesis of Compounds 1 and 2^a

^a Reagents and conditions: (a) NaBH₄, MeOH, rt, 30 min; (b) NaH (60%), DMF, 0 °C to rt, 1 h then methyl 5-bromovalerate, 0 to 70 °C, 12 h; (c) NH₂OH·HCl, KOH, MeOH, rt, 15 min; (d) LiOH, THF, MeOH, H₂O, 0 °C to rt, 1 h; (e) HCl·NH₂(CH₂)₄COOMe, EDCl, HOBT, DIEA, DMF, 0 °C to rt, 12 h.



Scheme 3. Synthesis of Compounds 3–11^a

^a Reagents and conditions: (a) LiOH, THF, MeOH, H₂O, 0 °C to rt, 1 h; (b)

HCl-NH₂(CH₂)_nCOOMe, EDCl, HOBT, DIEA, DMF, 0 °C to rt, 12 h; (c) NH₂OH·HCl, KOH, MeOH, rt, 15 min; (d) TFA, CH₂Cl₂, 0 °C to rt, 2 h; (e) Ac₂O, pyridine, CH₂Cl₂, 0 °C to rt, 5 h.

Table 1
In Vitro HDAC Enzyme Inhibitory Activities of Compounds 1–11 and SAHA

compd	ClogP ^d	IC ₅₀ (nM)													
		class I					class II								
		HDAC1	HDAC2	HDAC3	HDAC8	HDAC10	HDAC6	HDAC9	HDAC11	HDAC12	HDAC13	HDAC14			
1	1.12	124 ± 3	133 ± 4	12.9 ± 0.3	3320 ± 282	49.5 ± 2.9	155 ± 3								
2	1.19	644 ± 19	1690 ± 49	158 ± 9	3580 ± 181	61.9 ± 4.7	852 ± 30								
3	3.29	100 ± 16	238 ± 3	<0.002	8104 ± 681	<0.002	28.2 ± 5.4								
4	2.31	826 ± 33	2980 ± 97	83.6 ± 6.4	5810 ± 337	3.98 ± 0.48	891 ± 34								
5	1.25	20.7 ± 3.2	56.7 ± 3.4	4.04 ± 0.18	938 ± 43.6	2.54 ± 0.22	17.5 ± 1.2								
6	0.27	716 ± 32	1940 ± 71	144 ± 10	3430 ± 151	72.2 ± 5.6	840 ± 37								
7	3.29	271 ± 23	252 ± 10	0.42 ± 0.08	6851 ± 707	0.002	90.7 ± 12								
8	2.31	628 ± 23	1740 ± 77	115 ± 6	3650 ± 196	31.6 ± 1.6	690 ± 27								
9	1.25	56.3 ± 4.9	147 ± 4	10.2 ± 0.4	3458 ± 259	5.86 ± 0.16	44.9 ± 9								
10	0.27	360 ± 12	1050 ± 32	65.8 ± 2.1	2830 ± 128	35.1 ± 2.0	401 ± 10								
11	1.28	64.5 ± 3.6	131 ± 6	18 ± 1	4900 ± 253	6.81 ± 0.28	73 ± 3								
SAHA ^a	1.44	68 ± 14	164 ± 45	48 ± 17	1524 ± 463	90 ± 26	<i>b</i>								
SAHA ^c	1.44	96	282	17	1140	14	72								
TSA	2.23	4	14	2	1.38	1	5								

^aSAHA data from ref 9.

^bNot available.

^cSAHA and TSA data provided by Amphora Inc.

^dThe partition coefficients (ClogP) were calculated according to the fragment-based program KOWWIN 1.63 http://www.syrres.com/esc/est_kowdemo.htm.

Table 2
In Vitro Antiproliferative Activity of Compounds 1 – 11 against Pancreatic Cancer Cell Lines

compd	IC ₅₀ (μM)									
	BxPC-3	HupT3	Mia Paca-2	Panc 04.03	SU.86.86	HMEC ^a	HPDE6c7 ^a			
1	7	0.4	1	1.5	1	<i>b</i>	<i>b</i>			
2	28	5	4	5	5	<i>b</i>	<i>b</i>			
3	1	0.8	0.7	1	1	7	1.5			
4	36	1.4	8	14	22	<i>b</i>	<i>b</i>			
5	0.7	0.1	0.1	0.9	0.2	<1	0.2			
6	38	37	25	>50	25	<i>b</i>	<i>b</i>			
7	1	0.3	0.1	0.1	0.6	<1	0.5			
8	25	10	12	16	24	<i>b</i>	<i>b</i>			
9	0.8	0.2	0.1	0.1	0.8	<1	0.4			
10	33	7	10	17	11	<i>b</i>	<i>b</i>			
11	6	1	0.9	4	1	<i>b</i>	<i>b</i>			
SAHA	5	0.8	1	1	1	1.3	1.3			

^aNormal cell lines.

^bNot detected.