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Targeting epigenetic reader and eraser: Rational design, synthesis and in vitro evaluation of dimethylisoxazoles derivatives as BRD4/HDAC dual inhibitors

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ABSTRACT

The bromodomain protein module and histone deacetylase (HDAC), which recognize and remove acetylated lysine, respectively, have emerged as important epigenetic therapeutic targets in cancer treatments. Herein we presented a novel design approach for cancer drug development by combination of bromodomain and HDAC inhibitory activity in one molecule. The designed compounds were synthesized which showed inhibitory activity against bromodomain 4 and HDAC1. The representative dual bromodomain/HDAC inhibitors, compound **11** and **12**, showed potent antiproliferative activities against human leukaemia cell line K562 and MV4-11 in cellular assays. This work may lay the foundation for developing dual bromodomain/HDAC inhibitors as potential anticancer therapeutics.

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In the past few years, epigenetic modulators have emerged as promising targets for therapeutic development.[1] Targeting epigenetic modifiers and readers, particularly histone deacetylases (HDACs) and bromodomain-containing proteins (BCPs) have recently set the foundation for a new generation of anti-cancer drugs.[2–4] Histone lysine acetylation (KAc) is an epigenetic mark associated with gene activation. These acetyl groups are reversibly maintained by histone acetyltransferases (HATs) and histone deacetylases (HDACs).[5–7] HDACs as epigenetic 'eraser' modules catalyse the removal of acetyl groups from lysine residues on the histone tail to induce a condensed chromatin structure, and thereby inhibit transcription. HDACs play key roles in coordinating the interaction of intracellular signaling pathways with chromatin remodeling and transcription factor function to specifically mediate gene expression during cellular activation, proliferation, and differentiation.[8,9] Mounting evidence points to a link between dysregulated HDAC activity and many oncologic and nononcologic diseases.[10–12] Several small molecule HDAC inhibitors have been approved by FDA for the treatment of a variety of haematological and solid tumors, such as the hydroxamate containing inhibitor vorinostat (SAHA, **1**)[13] (Fig. 1) and more types have entered clinical trials.[14]

Histone acetylation also promotes transcription by binding to proteins involved in gene activation such as BCPs. The dysregulation of the bromo and extraterminal (BET) family of BCPs, which includes BRD2, BRD3, BRD4 and BRDT, has been implicated in the development of cancers such as NUT midline carcinoma and AML. Inhibitors of BET proteins (BETi) can block cancer cell proliferation and induce apoptosis in a wide range of tumor types.[15,16] To date, many effects of BETi have been attributed to transcriptional suppression of genes like the MYC oncogene. Specifically, BET inhibitors showed strong efficacy in AML models as well as a nut midline carcinoma model. Several distinct chemotypes, such as methyltriazolodiazepines (**2**)[17] and 3,5-dimethylisoxazoles (**3** and **4**)[18,19] (Fig. 1), have been identified.

Studies have suggested that BET and HDAC inhibitors induce similar genetic and biological effects and synergize to kill Myc-induced murine lymphoma.[20] The synergistic and therapeutic combinations by targeting the genetic link between BETi and HDACi will be a feasible approach to cancer drug development. Atkinson et al.[21] reported a dual active BRD/HDAC small molecule probe by the fusion of a BRD active tetrahydroquinoline (THQ) core

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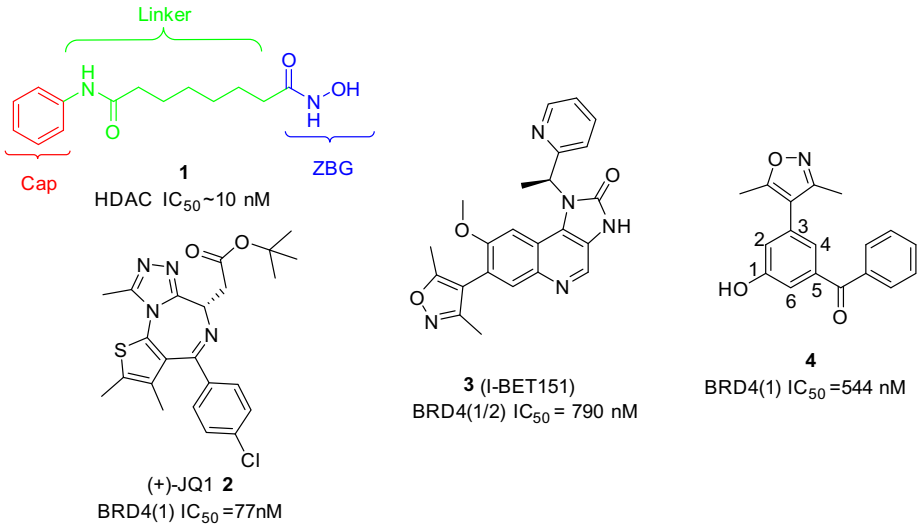

Figure 1. Structures of representative HDAC inhibitors (**1**), BRD inhibitors (**2**, **3** and **4**) with IC_{50} values.

with a hydroxamic acid HDACi motif. This demonstrated that dual BET/HDACs inhibitors can be rationally designed for disease applications. However, obtaining potent, selective BET/HDACs dual inhibitors still need further investigation.

Recently, dimethylisoxazole has been proved to be an effective scaffold of BETi. I-BET151 (**3**), a representative of this series, displays outstanding pharmacokinetic properties, such as bioavailability and half-life.[22] This suggested that dimethylisoxazole derivatives as a good starting point are worth further exploring. Investigating structural requirements of BRD4 and HDAC inhibitors led us to design and synthesize a series of novel 3,5-dimethylisoxazole derivatives as BRD4/HDAC dual inhibitors. These compounds were evaluated for inhibitory activities toward both HDAC1 and BRD4 with selectivity versus other bromodomain-containing proteins, and anti-proliferative activity against human chronic myelogenous leukemia (CML) cell line K562.

The rationale for the dual-inhibitor design originated from our deep insights into previously reported X-ray crystal structure of **4** bound to human BRD4 (Fig. 2A) and SAHA bound to a HDAC homolog (Fig. 2B). The 3,5-dimethylisoxazole of **4** occupies the KAc-binding pocket, acting as a KAc mimic, with isoxazole oxygen atom forming a hydrogen bond with the side chain NH_2 of N140 and the isoxazole nitrogen atom interacting with the conserved tyrosine Y97 via the bridging water molecule. Moreover, the benzoyl moiety of **4** well occupied the WPF shelf. It is notable that the ZA channel, a hot spot region for the design of BRD4 inhibitors, has not been fully occupied by the hydroxyl group of **4**. The hydroxyl offers a vector projecting toward the ZA channel, where flexible chain-like groups may be favorable. The long alkyl chain of SAHA located in a long narrow tubular channel. Hydroxamate forms three hydrogen bonds with Y297, H131 and H132, respectively. The capping phenyl group binding to the hydrophobic part on the protein surface. It is well established that the pharmacophore of HDACi (Fig. 1, compound **1**) consists of a capping group, an appropriate linker and a zinc-binding group (ZBG). Generally, ZBG plays a significant role in the binding efficiency between HDACi and enzyme. Hydroxamate is one of the most potent zinc ion chelating group among all types of ZBGs. Given the known binding modes of the BET inhibitor **4** and SAHA, it was envisaged that amalgamation of these pharmacophores would be a viable strategy to single molecules retaining both activities. We postulated that introduction of the linker and ZBG of HDACi SAHA at

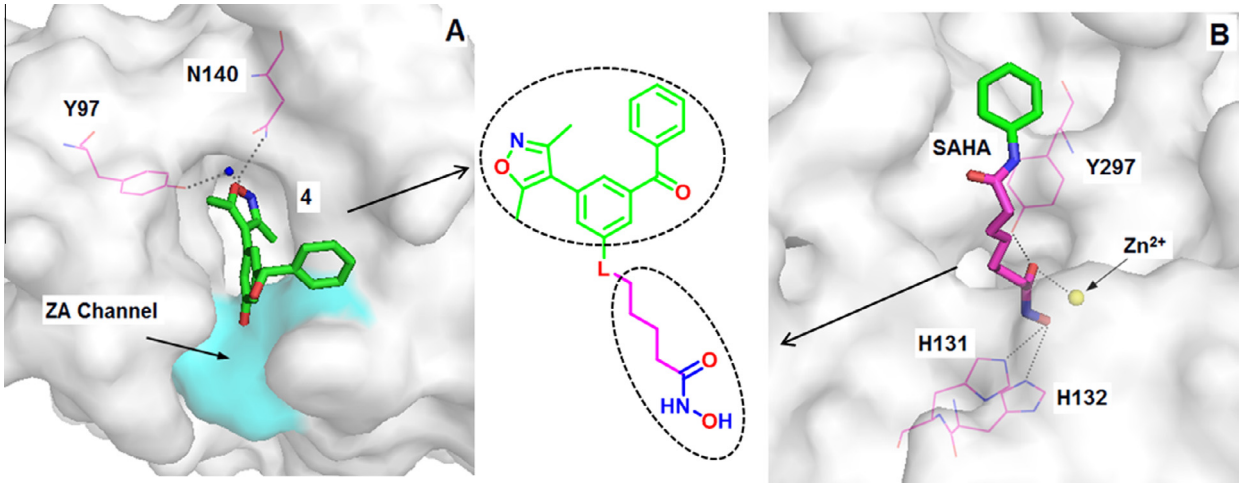

Figure 2. Design of dual inhibitors of BRD4/HDAC (A) X-ray crystal structures of **4** bound to human BRD4 (PDB entry: 4J0S), (B) SAHA bound to a HDAC homolog (PDB entry: 1C3S). The proteins are shown as white surface or cartoon. **4** and SAHA are shown in sticks. Water molecule is shown as blue spheres, and the hydrogen bonds were denoted by black dash lines. Figures were prepared using PyMOL.

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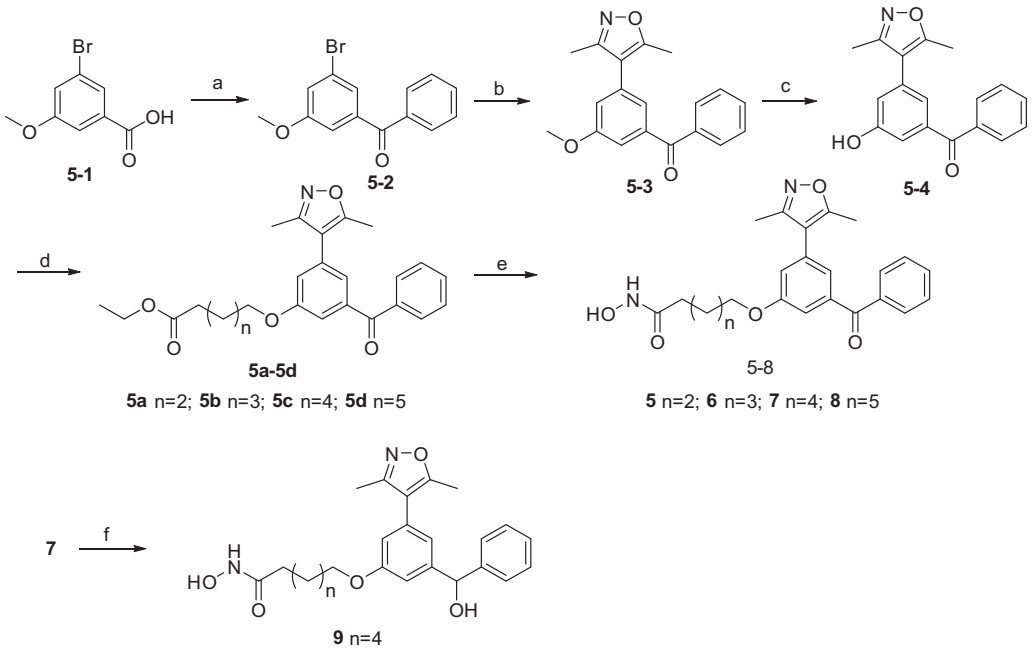
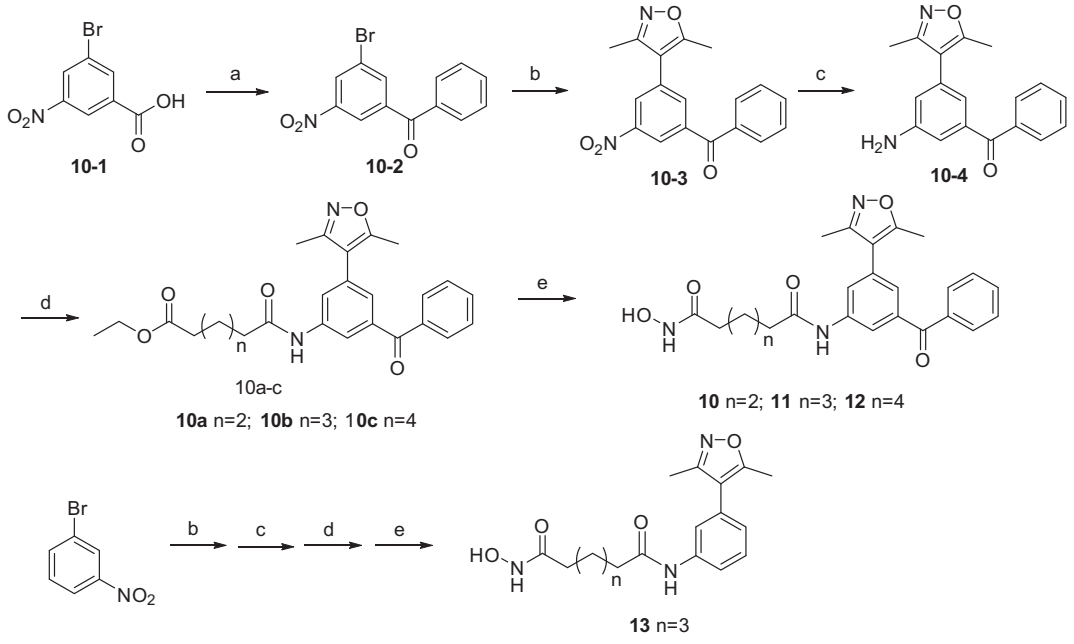
the position 1 of **4** would be compatible with HDAC inhibition since that the ZA channel of Bromodomain and the tubular channel of HDAC share considerable similarities (Fig. 2). In the meantime, the linker and ZBG of SAHA may direct into the ZA channel to make it well occupied which may increase the affinity to BRD4. The fused molecules were expected to retain the essential interactions with both proteins to exert desired biological functions.

The synthetic routes of **5–9** and **10–13** were outlined in Schemes 1 and 2. Synthesis of intermediate **5–4** started with the Friedel–Crafts acylation of benzene with 3-bromo-5-methoxybenzoic acid **5–1** to give benzophenone **5–2**. The 3,5-dimethylisoxazole moiety was introduced to **5–2** by coupling reaction to afford **5–3**. Treatment of compound **5–3** with tribromoborane produced the key intermediate **5–4**. The refluxing of **5–4** with alkyl bromides in the presence of anhydrous K_2CO_3 and tetrabutylammonium iodide in acetone afforded **5a–5d**. Finally, the ethyl ester groups in compounds **5a–5d** were treated with freshly prepared hydroxylamine in methanol to produce target compounds **5–8**. Similar chemistry was applied to the synthesis of **10–13** in Scheme 2. Hydrogenation of compound **10–3** using Pd/C under an atmosphere of hydrogen yielded amine **10–4**. Subsequent amidation of amine **10–4** with the corresponding carboxylic acid afforded compounds **10–13**.



Scheme 1. Synthesis of Compounds **5–9**. Reaction conditions: (a) $SOCl_2$, Benzene, $AlCl_3$, 75%; (b) 3,5-dimethylisoxazole, $PdCl_2$, AcOK, DMAc, 71%; (c) BBr_3, CH_2Cl_2, 81%; (d) tetrabutylammonium iodide, K_2CO_3, alkyl bromide, 68–75%; (e) MeOH, NH_2OH in MeOH, 86–91%; (f) $NaBH_4$, THF, 90%.



Scheme 2. Synthesis of Compounds **10–13**. Reaction conditions: (a) $SOCl_2$, Benzene, $AlCl_3$, 69%; (b) 3,5-dimethylisoxazole, $PdCl_2$, AcOK, DMAc, 94%; (c) H_2, Pd/C, MeOH, 95%; (d) EDCI, HOBT, carboxylic acid, 81–87%; (e) MeOH, NH_2OH in MeOH, 82–86%.

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Table 1
IC_{50} values of compounds ($n = 3$) **5–13** against BRD4 and HDAC1

Compd	IC_{50}(μM)	
	HDAC1	BRD4
5	5.45	1.49
6	1.49	1.38
7	1.43	1.05
8	1.74	2.99
9	0.97	0.77
10	0.76	0.78
11	0.27	0.85
12	0.15	0.67
13	0.36	3.73
MS275	0.14	n.d.[a]
JQ1	n.d.[a]	0.09

[a] n.d. = not determined.

Table 2
The temperature shifts (T_m) of compounds ($n = 3$) **5–13** against diverse BRDs under 25 μM

Compd	Temperature shifts (ΔT_m)					
	BRD4	BRD9	CECR2	CREBBP	PCAF	TAF1
5	5.89	−0.75	n.d.[a]	−0.01	−7.9	1.14
6	7.54	1.56	1.18	1.69	0.28	1.69
7	7.41	1.72	n.d.	2.13	1.04	2.22
8	4.91	1.22	2.06	0.65	−0.31	1.77
9	7.53	1.55	1.32	4.03	0.35	1.72
10	6.21	2.79	1.65	2.05	0.31	1.95
11	6.37	2.26	1.52	2.32	0.92	1.76
12	7.49	2.97	1.96	3.24	1.35	2.22
13	3.58	−0.13	0.64	0.73	0.05	1.08
JQ1	9.23	−0.22	1.14	0.07	0.79	−0.05

[a] n.d. = not determined.

Table 3
Antiproliferative activities of **11** and **12**

Compd	IC_{50} (μM)	
	K562	MV4-11
11	2.33	1.04
12	1.86	0.91
JQ1	0.64	0.24
SAHA	0.17	0.72

To study the biological activity, compounds **5–13** were evaluated against BRD4 activity assays with JQ1 as a positive control and HDAC1 enzyme assay with MS275 as a positive control. As shown in Table 1, most compounds exhibited moderate to good inhibitory activity against both BRD4 and HDAC1. The length of the hydroxamic acid side chains of this series have marginal effects on HDAC1 inhibition (6, 7 > 8 > 5) and the optimal carbon chain length is five or six carbon atoms. Diversity length of the side chains are tolerable to BRD4 inhibition due to the long ZA channel in BRD4. The linker between the hydroxamic acid side chain and the phenylisoxazole skeleton has relatively significant effect on both BRD4 and HDAC1 inhibition. Compounds with an amide group as the linker are more potent than those with oxygen atoms. The activity against BRD4 declined when removing the benzoyl moiety from **11**. This is probably because there is no fragment in **13** to occupy the WPF shelf in BRD4. Among the compounds tested, **12** showed powerful inhibitory activity against HDAC1 (IC_{50} = 0.15 μM) and BRD4 (IC_{50} = 0.67 μM).

The biology is still not well understood for many bromodomains; therefore, contamination of off-target bromodomain activity can easily confound results. Consequently, gaining selectivity against the BET bromodomains has been of utmost importance because inhibition of BET produces very strong and broad phenotypes.[23] Fragments in the ZA channel may contribute to the selectivity of inhibitors due to less conservative residues at this region. We selected all nine compounds to profile the binding specificity in six representative bromodomain modules, including BRD4, BRD9, CECR2, CREBBP, PCAF and ATAD2 by a thermal melting assay based on protein stability shift (Table 2). Of compounds that produced substantial changes in melting temperature (T_m), all displayed selectivity for BRD4.

BET bromodomain and HDAC inhibitors have previously shown antiproliferative effects in a variety of hematopoietic malignancies, including myelogenous leukemia and multiple myeloma. Consequently, three most potent compounds **10–12** were chosen as representatives to evaluated antiproliferative activities against human chronic myelogenous leukemia (CML) cell line K562 and human acute myelogenous leukemia (AML) cell line MV4-11. The IC_{50} values of compounds **10–12** were shown in Table 3. As expected, compounds **11** and **12** exhibited remarkable effects on antiproliferative activities in vitro, and comparable to the positive control JQ1.

Compound **12**, the most potent compound of this series, was chosen as a representative for docking into the BRD4 (1) and

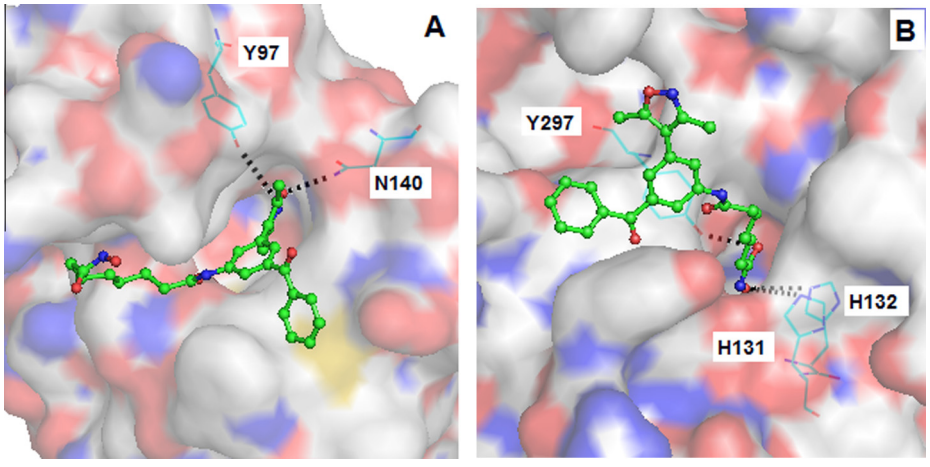

Figure 3. The docking model of **12** bound to human BRD4(1) (PDB entry 3MXF) and HDAC1 (PDB entry 1C3R). (A and C) The protein is shown as surface. **12** is shown in stick with carbon atoms colored in green, oxygen atoms in red and nitrogen atoms in blue. Water molecules are shown as pink spheres, and the hydrogen bonds were denoted by black dash lines. Figures were prepared using PyMOL.

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HDAC1, revealing excellent shape complementarity between ligand and the binding pocket. As shown in Figure 3, **12** binds to the BRD4 (1) as expected. The 3,5-dimethylisoxazole of **12** acts as an AcK mimic, with the methyl group occupying the hydrophobic pocket that recognizes the methyl group of AcK. The isoxazole oxygen atom forms the expected hydrogen bond with N140, and the isoxazole nitrogen atom interacts with the side chain Y97 via a water molecule. The benzoyl moiety well occupied the WPF shelf. The long alkyl chain oriented toward the ZA channel. The docking mode of **12** in complex with HDAC1 reveals that this compound binds in the HDAC1 pocket in a similar manner to SAHA and forms same interactions as SAHA. The hydroxamic acid group anchors **12** into the active site by chelating the essential catalytic zinc ion. Hydroxamate forms three hydrogen bonds with Y297, H131 and H132, respectively. The long alkyl chain of **12** exits the reaction centre along the long narrow tubular channel. This places the moiety from **4** in an open environment, weakly contacting hydrophobic residues on the protein surface.

In conclusion, using structure-based design approach, we have successfully generated a novel series of 3,5-dimethylisoxazoles with a hydroxamate group essential for chelation with the zinc ion in the active site of HDAC as novel BRD4/HDAC dual inhibitors. As expected, most of compounds exhibited distinct high to moderate inhibitory activity against both HDAC1 and BRD4. Moreover, the selectivity assay of all the compounds shows that they are excellent selective inhibitors for BRD4. In summary, we have demonstrated the example of dual-action inhibitors targeting the 'epigenetic eraser' HDAC and 'epigenetic reader' BRD as a promising approach to search for efficient anticancer multi-target agents.

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Supplementary data

Supplementary data (general biological methods and chemical experimental procedures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.04.034.

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