Letter

New dual CK2/HDAC1 inhibitors with nanomolar inhibitory activity against both enzymes

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New dual CK2/HDAC1 inhibitors with nanomolar inhibitory activity against both enzymes

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ABSTRACT: Four potent CK2 inhibitors derived from CX-4945 are described. They are provided also of nanomolar activity against HDAC1, therefore having promising utility as dual-target agents for cancer. The linker length between the hydroxamic acid and the CX-4945 scaffold plays an important role in dictating balanced activity against the targeted enzymes. The seven-carbon linker (compound **15c**) was optimal for inhibition of both CK2 and HDAC1. Remarkably, **15c** showed 3.0 and 3.5 times higher inhibitory activity than the reference compounds CX-4945 (against CK2) and SAHA (against HDAC1), respectively. Compound **15c** exhibited micromolar activity in cell-based cytotoxic assays against multiple cell lines.

Cancer is a multifactorial complex disease that is caused by multiple dysfunctions in genes or pathways.[1] Combination therapies directed to two or more molecular targets have been widely used in the treatment of this disease.[2] Nevertheless, combination therapy has serious disadvantages such as patient incompliance, difficulty to predict side effects and to optimize the dose ratio, as well as unwanted drug-drug interactions, and unpredictable pharmacokinetics.[2] Multi-target single agents are expected to improve the efficacy of the treatments, by exploiting synergistic interactions, avoiding problems of drug-drug interactions, decreasing drug resistance, and making the pharmacokinetic studies easier to perform.[3]

Histone deacetylases (HDACs) are a family of epigenetic enzymes that control the transcription and regulation of genes as well as cell proliferation, differentiation, migration, death, and angiogenesis.[4-6] Also, overexpression of HDACs has been found in many human cancers.[7] Therefore, inhibiting HDACs have been recognized as a promising approach for treating cancer. To date, four HDAC inhibitors (HDACi) were approved. by the FDA:[6] vorinostat (SAHA), romidepsin (FK-228), belinostat, (PXD101) and panobinostat (LBH589), and another one was approved by the Chinese FDA:[8] chidamide (tucidinostat, HBI-8000). On the other hand, almost 18 HDACi are in clinical trials.[6]

Protein kinase 2 (CK2) is an ubiquitously expressed and constitutively active serine/threonine kinase that phosphorylates an impressive array of substrates including HDACs.[9, 10] Overexpression of CK2 is involved in several human cancers and has also been linked to poor prognosis and disease progression.[11] Several CK2 inhibitors have been discovered in the past, but among them only two inhibitors, CX-4945 (NCT03904862) and CIGB-300 (NCT01639625) have recently entered into Phase II clinical trials as potential anticancer drugs.[12]

While many HDACi are in preclinical and clinical studies, they have been found to induce drug resistance[13] and their results in the treatment of solid cancers have been disappointing.[4-6] Interestingly, a SAR study confirms that the cap group in HDACi is flexible and tolerates modification. Thus, the zinc-binding group present in most of them can be easily linked to other anticancer scaffolds providing numerous multi-targeting agents with higher potency than the parent compounds.[4-6, 14, 15] Some examples are CUDC-101,[16] CUDC-907[17] and 4SC-202[18] (Figure 1) which have already entered clinical trials.[14] These results strongly support that a single compound that simultaneously inhibits HDAC1 and other oncological target could be beneficial in cancer resistance over single-acting agents, through the interference with multiple pathways.

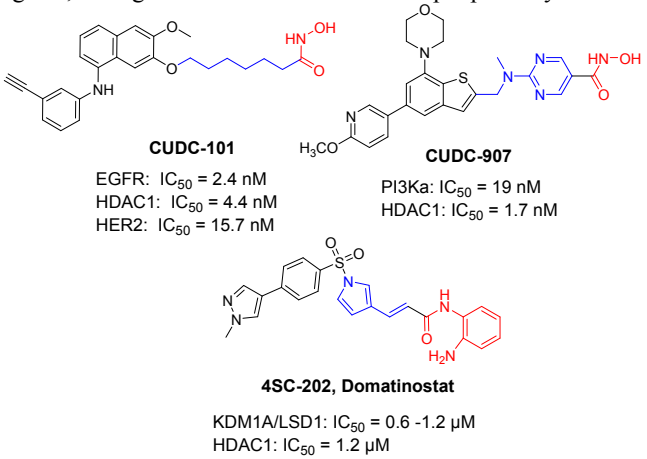


Figure 1. HDACi based dual inhibitors in clinical trials

Protein Kinase 2 (CK2) regulates the dimerization of HDAC1 and HDAC2 during mitosis[19]. Under hypoxic conditions, CK2 phosphorylates HDAC leading to HDAC activation. Activated HDAC contributes to tumor growth and vasculogenesis by Hippel-Lindau protein (pVHL) downregulation and, hence, to

the increased expression and stabilization of hypoxia-inducible factor HIF-1α protein.[20] Since both CK2 and HDAC are involved in the related cancer-relevant biological pathways,[21-24] we anticipated that simultaneously inhibiting these two targets by a multi-target single molecule should improve efficacy compared to single-target agents.

In previous publications,[25,26] we developed a series of dual CK2/HDAC1 inhibitors using TBB and DMAT as a scaffolds to promote CK2 inhibition and a hydroxamate zinc binding group (ZBG) to interact with the zinc present in the active site of HDAC1 and simulating the structure of SAHA, a potent inhibitor of HDAC1. The synthesized dual-acting agents exhibited promising inhibitory activities with the best compound showing IC_{50} of 5 μM for both enzymes and micromolar activity in cell-based assays (Figure 2).

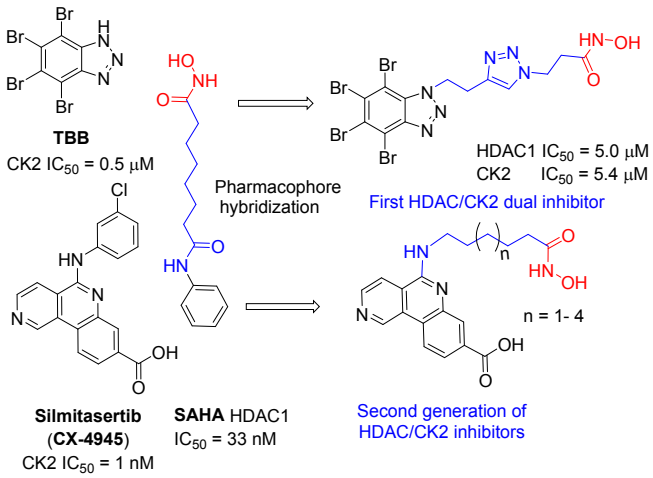


Figure 2. Design strategy for dual CK2/HDAC1 inhibitors.

Encouraged by these results, we have designed a new series of CK2/HDAC1 dual inhibitors where the TBB scaffold has been substituted by CX-4945, a 500- fold more potent CK2 inhibitor than TBB.[12, 27] Herein we describe the computational design and synthesis of these compounds, together with some promising preliminary assays of their biological activity.

RESULTS AND DISCUSSION

Computational docking and molecular dynamics

The binding to the target proteins was analyzed by means of docking and molecular dynamics (MD). The binding mode to CK2 of **15a-d** was similar to that of CX-4945 in the reference crystal structure[28] (PDB code 3PE1) (Figure S1, Supporting information). The carboxylic acid moiety interacts with the side chain of Lys68 and the backbone NH of Asp175; whereas, the pyridine nitrogen interacts with the backbone NH of Val116 in the hinge region. The linkers oriented the hydroxamic moieties towards the entrance of the nucleotide-binding site establishing different hydrogen bonds with the amino acids side chains depending on the length of the linker (Figure S2, Supporting information). The stability of the binding mode of compound **15c** (Figure 3) was assessed by a MD simulation that proved that, despite the high mobility of the linker, the initial binding mode and the interactions of the CX-4945 moiety were maintained, whereas the original hydrogen bonds established by the hydroxamic acid varied, but always maintained an interaction with the entrance of the binding site (Figure S3, Supporting information). These results demonstrate that the incorporation of an alkyl chain with a hydroxamic acid to the CX-4945 core does not impair the proper orientation of the molecules in the ATP binding site, as well as the establishment of the essential interactions that guarantee the high potency found for the reference compound CX-4945.

The binding of compounds **15a-d** to HDAC1 was similar to that of SAHA (Vorinostat®) to different HDACs in the reference crystal structures.[29,30,31] (Figure S4, Supporting information). All compounds established with HDAC1 a bidentate chelation to the catalytic Zn^{2+} ion stabilized by hydrogen bonds with the side chains of His140 and Tyr303, and oriented the CX-4945 moiety to establish different interactions with amino acids in the surface of the protein that also varied depending on the length of the linker (Figure S5, Supporting information). The stability of the binding mode of compound **15c** to HDAC1 (Figure 4) was also assessed by means of MD simulations. During the simulation the CX-4945 moiety explored the surface of the protein establishing different interactions through the carboxylic acid moiety but mainly establishing π-stacking interactions with the side chain of Phe205 (Figure S6, Supporting information). The movement brought about by the linker and the CX-4945 moiety destabilized some of the initial hydrogen bonding interactions between the hydroxamate moiety and the side chains of His140 and Tyr303; however, a new and stable hydrogen bond is established with the side chain of His141. These results suggested that the interaction between the CX-4945 cap and residue Phe205, and an optimal linker length to establish bidentate Zn^{2+} coordination is important for the HDAC1 inhibitory potency.

These results predict an overall good affinity of **15a-d** for CK2 and HDAC1, so they were forwarded for synthesis and enzymatic studies.

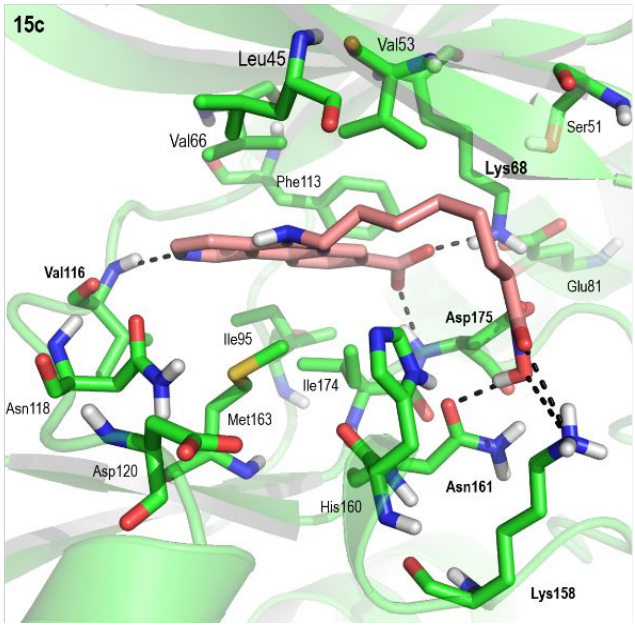


Figure 3. PyMOL stick and cartoon representation of the best docking pose of compound **15c** to CK2. For the sake of clarity,

only polar hydrogens are shown, and hydrogen bonds have been highlighted with dashed lines.

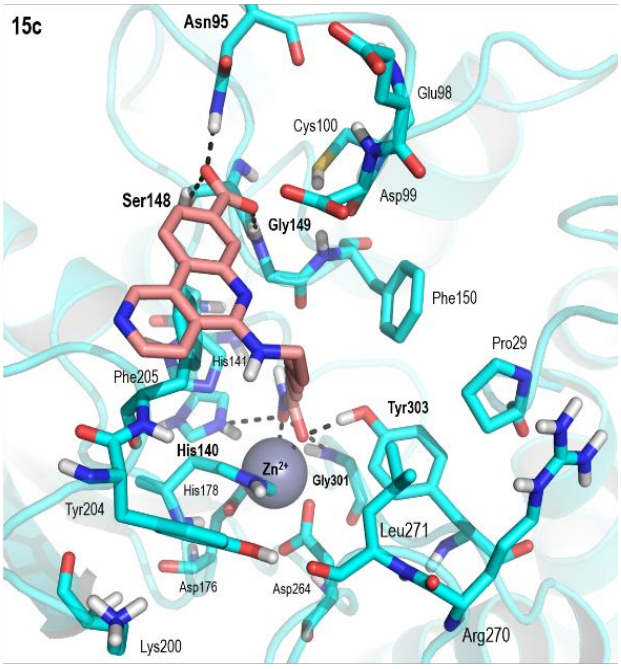
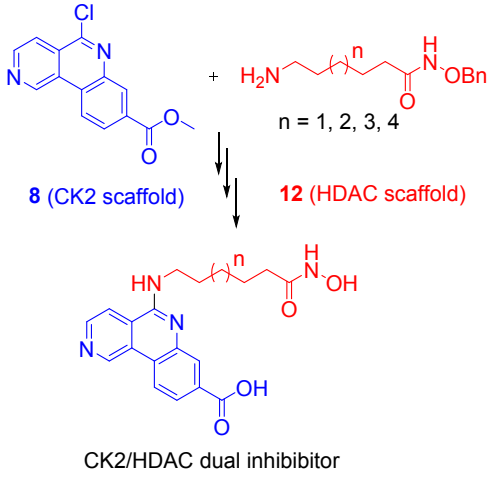


Figure 4. PyMOL stick and cartoon representation of the best docking pose of compound **15c** to HDAC1. For the sake of clarity, only polar hydrogens are shown, and hydrogen bonds have been highlighted with dashed lines.

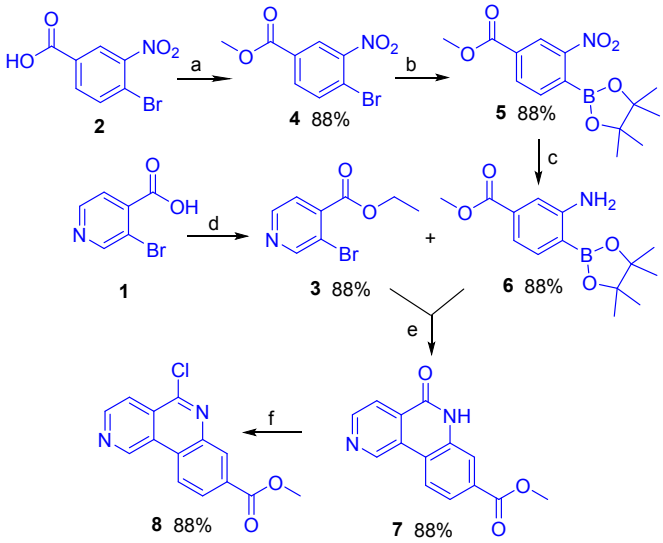
Chemistry

A convergent strategy was developed for the synthesis of this new generation of dual inhibitors. First, chloroquinoline **8** which possesses a chlorine atom that allows the substitution with different nucleophiles was synthesized. Then, different Ω–amine benzyloxy-protected compounds **12a-d** were selected and synthesized in order to assess the linker that best fits in the catalytic site of HDAC1 (Scheme 1).



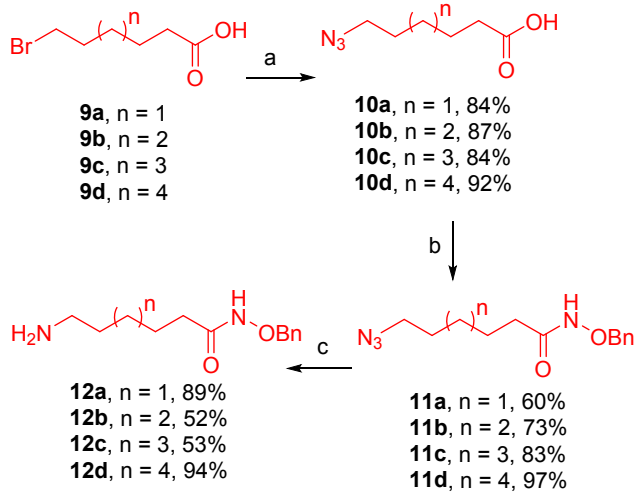
Scheme 1. Convergent strategy for dual inhibitor synthesis.

Compound **8** was efficiently synthesized adapting the procedure described in the literature[32] (Scheme 2). Compounds **3** and **6** were synthesized from commercially available carboxylic acids **1** and **2** as described in Scheme 2. Then, lactam **7** was obtained through a one-pot Suzuki coupling/intramolecular amide cyclization between the boronic ester **6** and the ethyl ester **3**. Treatment of lactam **7** with phosphorous oxychloride afforded chloroquinoline **8** which was ready for the substitution with the different amines **12a-d**.



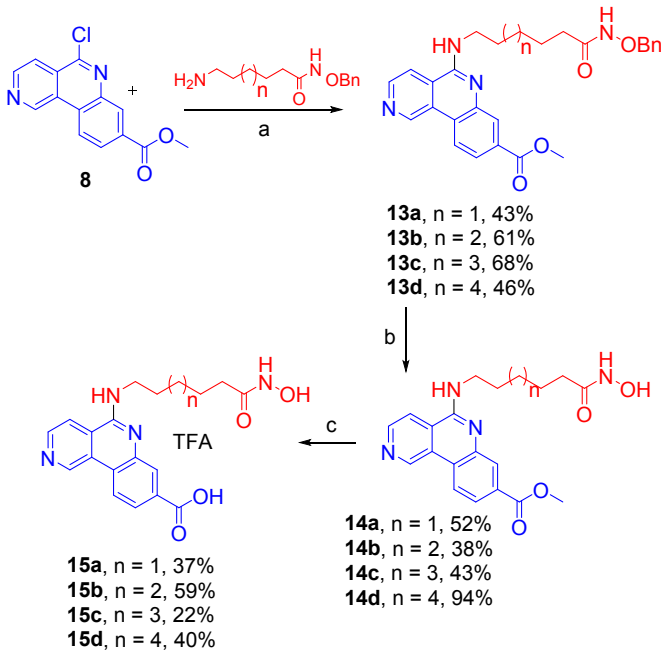
Scheme 2. Synthesis of compound **8.**
Reagents and conditions: a) MeOH, H_2SO_4, reflux, 12 h; b) KOAc, $Pd(dppf)Cl_2$, Bis(pinacolate)diboron, DCM, Dioxane, 80 ºC, overnight; c) H_2, Pd/C 10%, EtOAc, RT, 12 h; d) MeOH, H_2SO_4, reflux, 12 h; e) NaOAc, $Pd(dppf)Cl_2$, DMC, Dioxane, 125 ºC, 12 h; f) neat $POCl_3$, reflux, 2h.

Amines **12a-b** were prepared as depicted in scheme 3. Nucleophilic substitution of the commercially available Ω–bromo carboxylic acids **9a-d** gave the corresponding azido carboxylic acids **10a-d**. Amide coupling of **10a-d** with the *O*-benzyl-hydroxylamine followed by selective catalytic hydrogenation of the formed azides **11a-d,** gave the desired amines **12a-d**. The use of ethyl acetate as solvent is the key condition to achieve selective reduction of the azide, without deprotecting the benzyl group present in the molecule. When more polar solvents such as methanol were used both reductions occurred.



Scheme 3. Synthesis of Ω–amine (benzyloxy)amino compounds **12a-d** as HDAC1 scaffold.
Reagents and conditions: a) NaN_3, DMF, 77 ºC, 48 h; b) HCl.H_2N-OBn, HBTU, DIPEA, DMF, RT, 12 h; c) H_2, Pd/C, EtOAc, RT, 2 h.

Finally, as depicted in scheme 4, chloroquinoline **8** and amines **12a-d** were reacted to yield methyl benzo[c][2,6]naphthyridine-8-carboxylates **13a-d**. After the *O*-debenzylation by catalytic hydrogenation at high pressure in methanol, these compounds were further converted to the corresponding carboxylic acids **15a-d** that were purified by HPLC and isolated as trifluoroacetic salts with the necessary purity (>95%) for biological assays.



Scheme 4. Synthesis of dual inhibitors **15a-d** isolated as TFA salts.
Reagents and conditions: a) DMF, K_2CO_3; 135 ºC, 55 min, MW irradiation; b) for **14a-c**: H_2, Pd/C 10%, MeOH, 4 bar, RT, 12 h, for **14d**: H_2 atmosphere and MeOH:THF 1:1 as solvent, 12 h; c) LiOH, RT, 96 h.

Enzymatic inhibitory evaluation

The inhibitory activities of the synthesized compounds were determined against CK2 and HDAC1 and the IC_{50} values are collected in Table 1. The activity towards human recombinant HDAC1 was tested using a fluorometric method.[25] CK2 inhibition was measured by a luminometric assay using ADP-Glo Kinase Assay (Promega).[33]

Table 1. Inhibitory activity (IC_{50}, nM) on HDAC1, HDAC6 and CK2

Cpd	HDAC1	HDAC6	CK2
15a	**190 ± 100**		**3.5 ± 3.3**
15b	**140 ± 20**		**14 ± 19**
15c	**3.3 ± 1.6**	**13 ± 1**	**1.7 ± 1.0**
15d	**130 ± 30**		**19 ± 24**
TSA	**5.6 ± 2.0**		
SAHA	**33.5**[34]	**33.1**[34]	
CX-4945			**1.8 ± 1.2**

The four compounds presented an inhibitory activity on CK2 in the low nanomolar range. This result demonstrates that the introduction of a carbon chain containing the hydroxamic group in the CX-4945 scaffold does not interfere in the interaction of these compounds with the active site of CK2 and supports our hypothesis that CX-4945 is an appropriate scaffold for the design of dual CK2/HDAC1 inhibitors.

The best result was found for **15c,** with a value of IC_{50} = 1.7 nM, similar to that obtained for the control compound (CX-4945). What is more interesting, **15c** is also a potent inhibitor of HDAC1, with a value of IC_{50} = 3.3 nM, which is 3.4-fold lower than the value obtained for SAHA in our hands. In the design of dual inhibitors, it is especially important to achieve a good balance in the activity against the two target enzymes. This is the case of **15c**, a promising compound with a remarkable potency for both enzymes.

Interestingly, this compound showed potent activity in HDAC6 (see table 1). SAHA is also highly potent against both HDAC1 (IC_{50}: 34 nM) and HDAC6 (IC_{50}: 33 nM) while is remarkably less potent in other isoforms such as HDAC4 (IC_{50}: >1000 nM) and HDAC8 (IC_{50}: 776 nM).[34]

When the chain connecting the two selected pharmacophores was shortened (compound **15b**) or elongated (compound **15d**) the inhibitory potency in CK2 was maintained, while some activity in HDAC1 was lost. These results demonstrated that the best length for a dual interaction is provided by a seven-carbon chain. Our efforts in the design of the next series of inhibitors will concentrate in the modification of the nature of the connecting chain, keeping the same length, and the modification of the ZBG.

Cytotoxic activity in cells

The cytotoxicity of the four compounds **15a-d** in comparison with reference compounds **SAHA** and **CX-4945** under identical conditions was investigated by using the resazurin assay.[35] In vitro cytotoxicity for CX-4945 was reported already against a panel of cancer cell lines, including LNCaP, PC3, MCF-7 and A549 and in vivo efficacy was tested in PC3 xenografts.[32] Similarly, the HDAC inhibitor SAHA showed low micromolar cytotoxicity against LNCaP, PC3, MCF-7 and A549 cell lines.[36-38] To directly compare the potency of our compounds with CX-4945 and SAHA, we selected these four human cancer cell lines, namely lung (A549), breast (MCF-7), and prostate (PC3 and LNCaP) cell lines (Table 2).

Table 2. IC_{50} values of compounds **15a-d**, **SAHA** and **CX-4945** against four human tumor cell lines.

Cpd	IC_{50} values (μM)			
	LNCaP	PC3	MCF-7	A549
15a	**>100**	**>100**	**>100**	**>100**
15b	**>100**	**>100**	**>100**	**>100**
15c	**16.31 ± 0.7**	**40.42 ± 4.4**	**52.48 ± 10.0**	**104.73 ± 7.9**
15d	**66.70 ± 5.0**	**>100**	**>100**	**>100**

SAHA	**0.69 ± 0.1**	**2.03 ± 0.1**	**1.26 ± 0.1**	**3.14 ± 0.2**
CX-4945	**6.52 ± 0.6**	**10.87 ± 0.6**	**9.41 ± 0.3**	**11.60 ± 1.8**

Interestingly, only **15c** showed activity in the cell experiments, which is in accordance with the docking and enzymatic assay results. The best activity was observed in LNCaP, an androgen-sensitive human prostate adenocarcinoma cell line, with an IC_{50} of 16.31 μM. The activity in this cell line was lower than the activities obtained for the parent compounds. Considering the results of inhibitory activity of **15c** in isolated enzymes, we postulate that the lower activity exerted by **15c** in cells could be due to a low permeability, which we will try to overcome in the next series of inhibitors.

In order to evaluate the effect on the cytotoxicity of the TFA salts,[39, 40] we carried out a cell assay on LNCaP cell line using CX-4945 as a TFA salt. At the concentrations used in the cytotoxicity study we observed slight differences compared to the free compound. (CX-4945 6.52 μM; CX-4945 as a TFA salt 3.70 μM).

In conclusion, we found four remarkably potent CK2 inhibitors, showing that the introduction of chains of different lengths provided with hydroxamate groups, does not affect the interaction of the CX-4945 ring with the active site of CK2.

The presence of the hydroxamate allows the interaction with HDAC1, providing only one promising dual CK2/HDAC1 inhibitory agent **15c,** with nanomolar activity in both enzymes. The modelling work has provided plausible binding modes that can account for these results.

Preliminary assays in human cell lines confirm the interest of this compound as a cytotoxic agent. Future studies are necessary to improve the pharmacokinetic profile of this new hit compound and examine its activity in other human cell lines.

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, spectroscopic and analytical data for all compounds; in vitro enzymatic assay, resazurin assay and computational methods are described.

The Supporting Information is available free of charge on the ACS Publications website.

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‡L.R and I. O contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

HDAC, histone deacetylase; CK2, Protein kinase type 2; SAHA, suberoylanilide hydroxamic acid (Vorinostat); TBB, 4,5,6,7-tetrabromo-2H-benzotriazole; DMAT, 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole; dppf, 1,1'-Bis(diphenylphosphino)ferrocene; DMF, dimethylformamide; MW, microwave; RT, room temperature; THF, tetrahydrofurane; ZBG, Zinc Binding Group; TFA, trifluoroacetic acid; TSA, Trichostatin A; HBTU, O-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; DIPEA, *N N*-Diisopropylethylamine; DCM, dichloromethane; THF, tetrahydrofurane; PDB, protein data bank; ATP, adenosine 5'-triphosphate.

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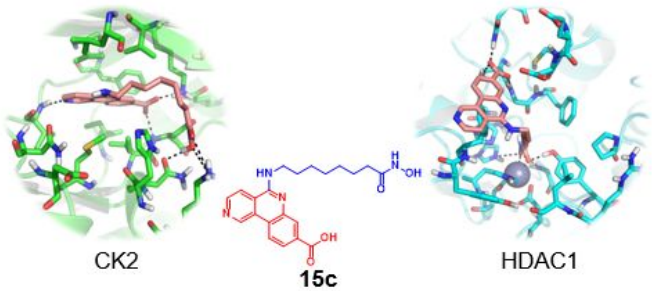

15c

CK2 IC_{50} = 0.6 nM
HDAC1 IC_{50} = 2.9 nM