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DESIGN, SYNTHESIS AND EVALUATION OF ANTIPROLIFERATIVE ACTIVITY OF MELANOMA-TARGETED HISTONE DEACETYLASE INHIBITORS

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Abbreviations: HDAC, Histone deacetylase; HDACi, Histone deacetylase inhibitors; ZBG, zinc binding group.

ABSTRACT:

The clinical validation of histone deacetylase inhibition as a cancer therapeutic modality has stimulated interest in the development of new generation of potent and tumor selective histone deacetylase inhibitors (HDACi). With the goal of selective delivery of the HDACi to melanoma cells, we incorporated the benzamide, a high affinity melanin-binding template, into the design of HDACi to generate a new series of compounds **10a-b** and **11a-b** which display high potency towards HDAC1 and HDAC6. However, these compounds have attenuated antiproliferative activities relative to the untargeted HDACi. An alternative strategy furnished compound **14**, a prodrug bearing the benzamide template linked via a labile bond to a hydroxamate-based HDACi. This pro-drug compound showed promising antiproliferative activity and warrant further study.

Melanoma is one of the most prevalent cancers among skin malignancies.[1] As with most cancer types caused by disruption in the homeostatic regulation of cellular processes, melanomas are typified by dysregulation of melanin-producing melanocytes by keratinocytes in the skin epidermis.[2] This dysregulation results in uncontrolled proliferation of melanocytes, hence the high melanin content associated with most melanoma cases. When detected early, melanoma can be corrected by surgery. Therefore, sustained efforts have been on developing effective new tools for early diagnosis of melanoma.

Over the past two decades, benzamides (Fig. 1A) have emerged as promising class of compounds that can be developed into theranostics for early detection and treatment of melanomas.[3] What makes this class of compounds suitable for this purpose is their high affinity for melanin, which affords them a high residence time in melanoma cells. In phase II clinical trial, *N*-(2-(diethylamino) ethyl)-4-iodobenzamide (Fig. 1A) showed 100% specificity on a lesion-site basis with little toxicity to the patients.[3a] When incorporated into the structure of a conventional alkylating agent, the resulting alkylating benzamides showed superior selectivity for melanoma cells both *in vitro* and *in vivo*.[4] Moreover, other studies have demonstrated the suitability of the benzamide template for developing targeted proteasome degradation inhibitors[5]. Hence, benzamides have become the Holy Grail for developing melanoma-targeted therapeutics.

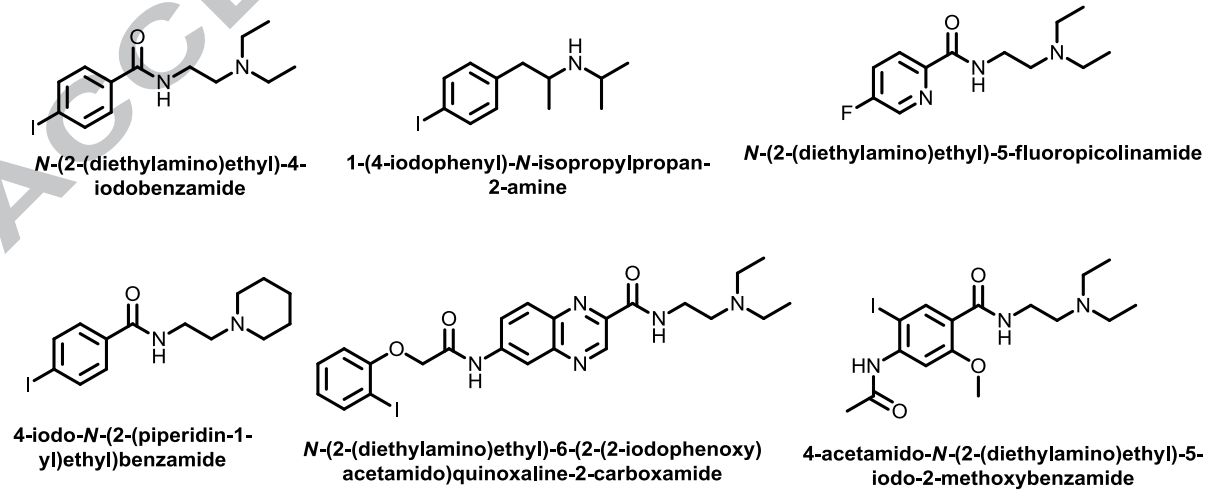
Epigenetic dysregulation, exemplified by aberrant regulation of expression of histone deacetylases (HDAC) and histone acetyl transferase (HAT), is one of the root causes of cancer. Several isoforms of HDAC have been implicated in the sustained proliferation of most tumors, with class I and class IIb HDACs being the most prominent.[6] With respect to melanomas, HDAC isoforms 5 and 6 were recently reported to play significant roles in disease progression.[7] To further exploit the relevance of HDAC 6 in melanoma progression, Kozikowski *et. al.*[8] have

studied the effects of HDAC 6 selective HDAC inhibitors (HDACi) on melanoma cell growth. Though a promising approach, these isoform 6 selective HDACi showed weak cytotoxic effects on B16F10 melanoma cell line.

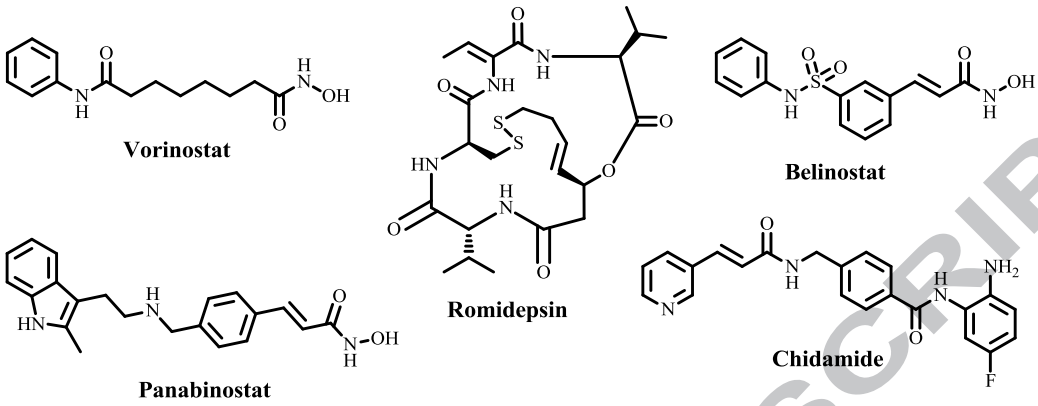
The structures of the five clinically approved HDACi (Fig. 1B) conform to a structural model comprising a surface recognition group, linker and a zinc binding group (ZBG) as shown for SAHA in Fig. 1C. Previous SAR studies on the surface recognition group have identified this part of most HDACi to be the most amenable to modification.[9]

To further demonstrate the broad scope of application of the benzamide template for developing targeted therapies, we designed and synthesized HDACi which have melanin binding benzamide group incorporated unto the surface recognition group (Figs. 2A and B). We envisaged that this approach will result in selective localization of benzamide-bearing HDACi within the melanoma cells, and mitigate the off-target toxicities associated with most HDACi. Following our previous reports,[9a] we restricted linker lengths in our design to be between 5-7 methylene groups separating the hydroxamate (ZBG) from the cap group.

A.



B.



C.

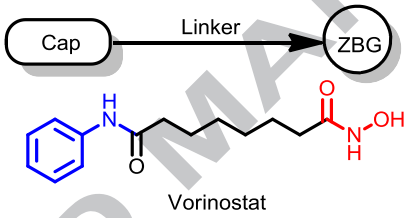


Fig. 1. **A**) structures of selected melanin-targeting benzamides for imaging melanomas[3b, 3c, 11]; **B**) structures of clinically approved HDACi; C) standard HDACi pharmacophoric model.

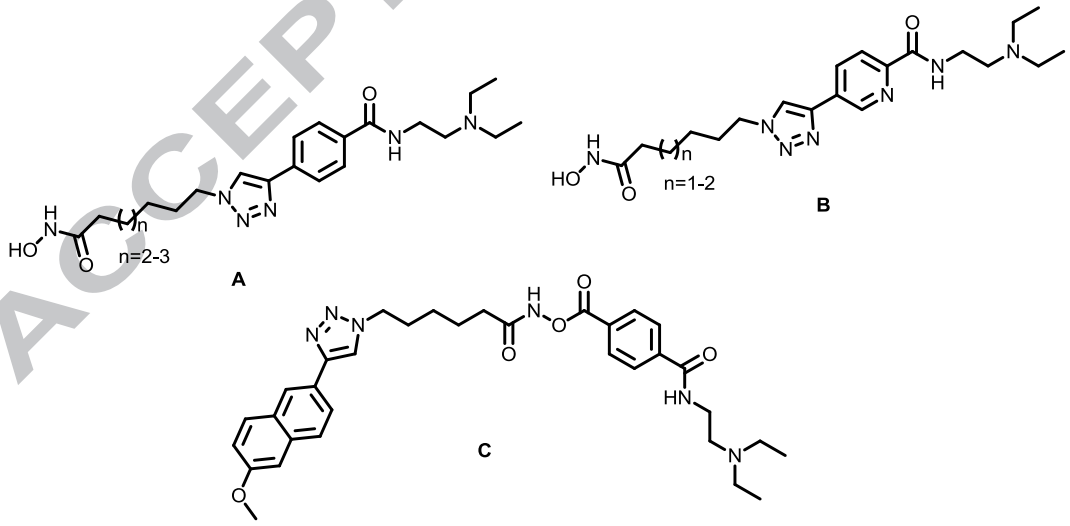
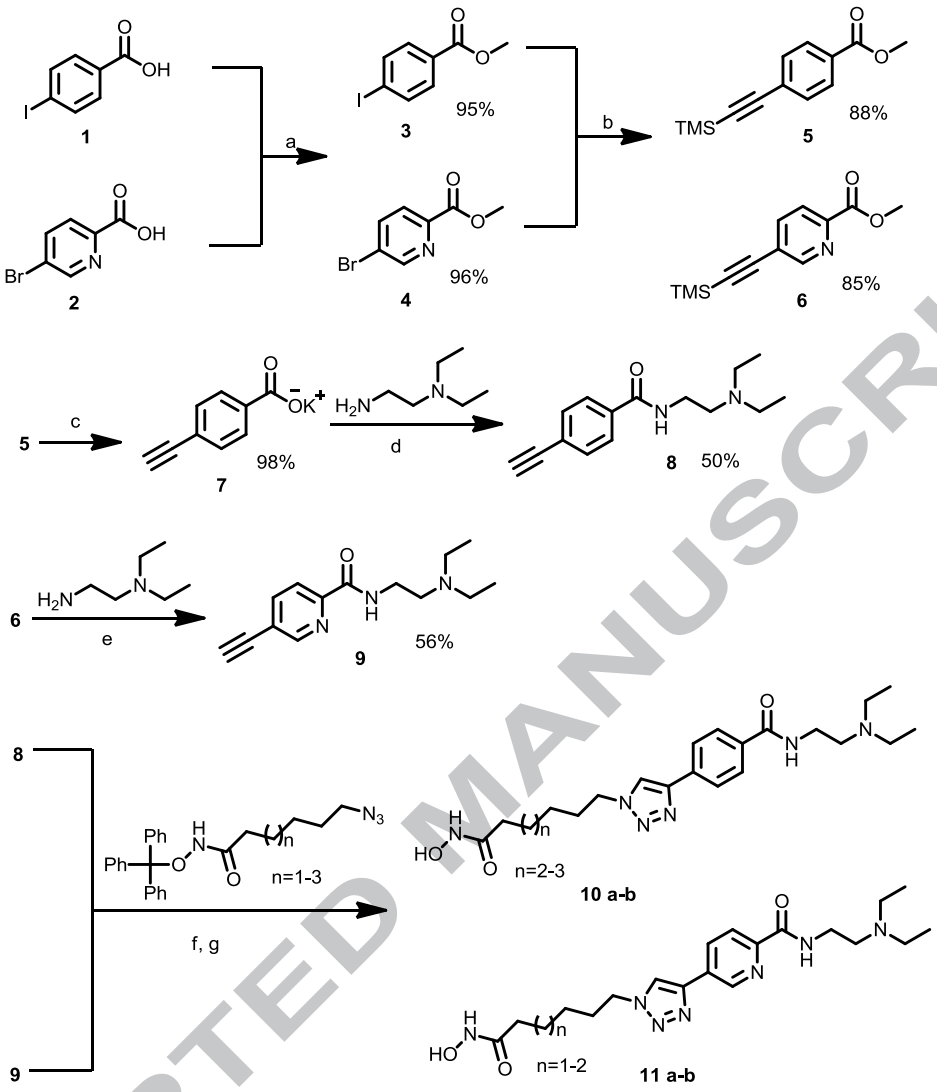


Fig. 2. General structures of designed compounds.

Synthesis of target compounds started with the esterification of 4-iodobenzoic acid **1** and 5-bromopyridine-2-carboxylic acid **2**, both of which are commercially, using TMSCl in MeOH to give methyl esters **3** and **4** (Scheme 1). Sonogashira coupling[12] of the respective halo-methyl ester (compound **3** or **4**) with TMS-acetylene gave the appropriate TMS-protected ethynyl intermediates **5** and **6**. At this stage, different synthetic pathways were employed to access alkynes **8** and **9**. Subjecting TMS-protected methyl-4-(ethynyl)-benzoate **5** to alkaline hydrolysis, using KOH in isopropanol, resulted in TMS removal and gave the potassium salt of 4-(ethynyl) benzoate **7**. Coupling of **7** to *N*,*N*-diethyl ethane-1,2-diamine using TSTU[3c] afforded compound **8**. In the case of compound **9**, TMS-protected methyl-5-ethynyl picolinate **6** was refluxed with *N*,*N*-diethyl ethane-1,2-diamine in MeOH to give compound **9**. Cu (I) catalyzed cycloaddition reaction[13] between alkyne **8** or **9** and *O*-trityl protected azido hydroxamates with appropriate linker lengths, followed by trityl deprotection using TFA, gave the target compounds **10a-b** and **11a-b**.

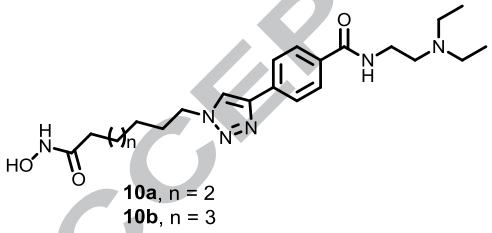


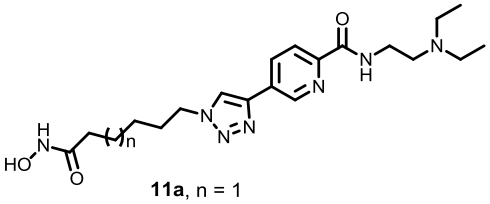
Scheme 1: Synthesis of Benzamide-bearing HDACi. a) TMSCl, MeOH, rt; b) TMS acetylene, $Pd(PPh_3)_2Cl_2$, CuI, TEA, CH_3CN, 90^0C, overnight; c) KOH, isopropanol, rt, overnight; d) TSTU, DIPEA,DCM, rt; e) MeOH, 120^0C; f) CuI, DIPEA,THF, rt; g) TFA/TIPS.

Taking into consideration the previously reported roles of HDAC6 in sustaining melanoma growth[7-8] and the absolute requirement of HDAC1 inhibition for HDACi to elicit robust antiproliferative effects,[8] we profiled the HDAC1 and HDAC6 inhibition activities of compounds **10a-b** and **11a-b** in a cell-free assay. All the compounds showed strong inhibitory

activities towards the two HDAC isoforms tested, with compound **10a** being ten-fold more potent towards HDAC1 compared to SAHA (Table 1). We observed these compounds to be more potent towards HDAC1 compared to HDAC6, suggesting that they may possess potent *in vitro* anticancer activities. The most potent compound in this series, compound **10a**, showed about four-fold selectivity for HDAC1 compared to HDAC6 while the weakest in the series, compound **11a**, is almost indistinguishable in terms of inhibitory activity towards HDAC1 and HDAC6. Compounds **10b** and **11b**, despite having seven- and six methylenes, respectively, as linkers are both equipotent towards HDAC1 and HDAC6. Interestingly, the phenyl-based compound with six methylene linker, **10a**, is more potent than the analogous pyridyl-based compound, **11b**, in both HDAC1 and HDAC6. This observation is similar to a previous study in our lab in which HDAC inhibitory activities of phenyl-based HDACi and their analogous pyridyl-based HDACi were compared.[14] Likewise, linker-length effect, similar to previous observations,[14-15] was noticeable in the two series of compound.

Table 1: HDAC isoforms inhibition study





Compound	n	IC_{50} (µM) *	
		HDAC1	HDAC6
10a	2	0.0038	0.016

10b	3	0.012	0.027
11a	1	0.100	0.170
11b	2	0.017	0.034
SAHA	-	0.031	0.011

*values indicate average of three independent experiments

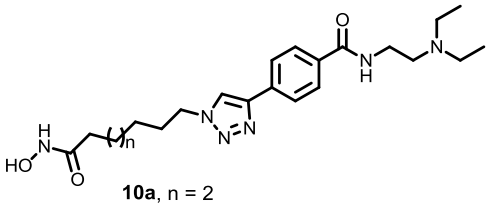
Encouraged by the potent cell-free HDAC inhibitory activities of these benzamide-bearing HDACi, we assayed their growth inhibitory effects on two melanin-producing melanoma cell lines, B16F10 and A375. We observed that compounds **10a** and **11b** were weakly cytotoxic towards the two cell lines while **10b** and **11a** were almost devoid of growth inhibitory effects at the maximum concentration tested (Table 2). The most active compound, **10a**, was about 3- and 6-fold less active relative to SAHA (control HDACi) against B16F10 and A375 cell lines respectively. This weak cytotoxic activity of these compounds against melanin-producing melanoma cell lines was surprising to us.

Previously, intricate drug efflux system in melanoma, involving melanosomal sequestration and subsequent efflux of cytotoxic agents, was identified to be responsible for resistance of melanoma to chemotherapeutic agents.[10, 16] It is quite possible that the weak cytotoxic effects observed with the benzamide-bearing HDACi in melanoma cell lines is a reflection of this phenomenon. Alternatively, melanin, being a non-biological polymer,[17] may be unable to release sufficiently high concentration of the benzamide-bearing HDACi upon binding, resulting in their inability to engage HDACs, the therapeutic targets. Hence, melanin binding may not provide the anticipated advantage. It is also possible that the observed weak

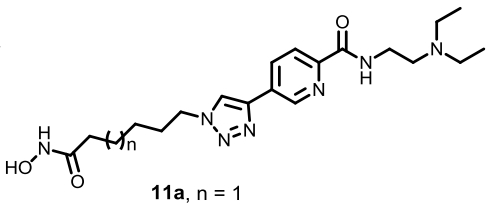
antiproliferative activity of the benzamide-bearing HDACi may just be a result of the inability of the tested compounds to penetrate the cell membrane.

Intrigued by this observation and determined to unravel the basis for the weak in cellulo activities of these compounds, we extended cell growth inhibition study to LNCaP, an androgen dependent prostate cancer cell line not known to produce melanin. Results obtained from this study should provide insights into the role (s) of melanin in the observed cellular inactivity, and also confirm the ability of these compounds to cross the cell membrane. As seen in Table 2, all of the tested compounds inhibited the growth of LNCaP in a pattern similar to the HDAC inhibitory activity, confirming that these compounds can cross the cell membrane easily. This makes it highly probable that either of melanin binding or effective drug efflux is impeding access to HDAC enzymes. To confirm the role of drug efflux on diminishing the cell growth inhibitory activity of these compounds, we treated B16F10 with compounds **10a** and **11b** followed by verapamil, an established efflux inhibitor,[18] at 100 µM. This verapamil co-treatment did not improve the cytotoxic effect of **10a** and **11b** in B16F10 (data not shown), strongly suggesting that the high affinity for melanin is the likely reason for the weak cytotoxicity of these compounds.

Table 2: Antiproliferation study in cancer cell lines.



10a, n = 2
10b, n = 3



11a, n = 1
11b, n = 2

Compound	n	IC_{50} (μM) *		
		B16F10	**A375**	**LNCaP**
10a	2	34.16	23.60	1.2
10b	3	75.91	>100	3.0
11a	1	>100	>100	10.5
11b	2	55.67	47.18	1.5
SAHA	-	13.1	4.1	1.8

*values indicate average of three independent experiments

We then considered exploiting benzamides' affinity for melanin in a slightly different way. For this, a prodrug approach, in which the melanin-binding benzamide is attached to the hydroxamate-moiety of a HDACi via a labile ester bond that could provide a means of selective delivery of such HDACi to melanoma, was developed. When such compound binds to melanin in melanoma cells, the HDACi component could, thereafter, be released by spontaneous hydrolysis and/or cellular esterase-facilitated hydrolysis. This approach has been successfully used in the past to improve the cellular accessibility of hydroxamate-based HDACi.[19] In addition to this; such a design could mask potential off target interaction of the hydroxamate group with other biological targets in the cells.

Previous work from our lab has identified triazolyl hydroxamate **7u** (scheme 2) as a promising HDACi with robust growth inhibitory activity in prostate cancer cell line. [14]

Encouraged by this, we incorporated compound **7u** into the design of a releasable benzamide-HDACi prodrug (Fig. 3).

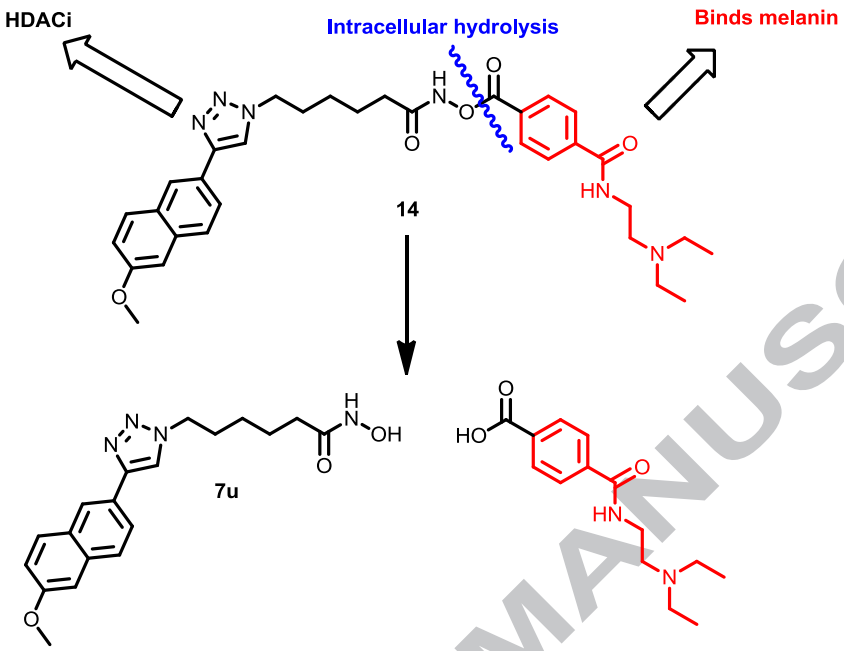
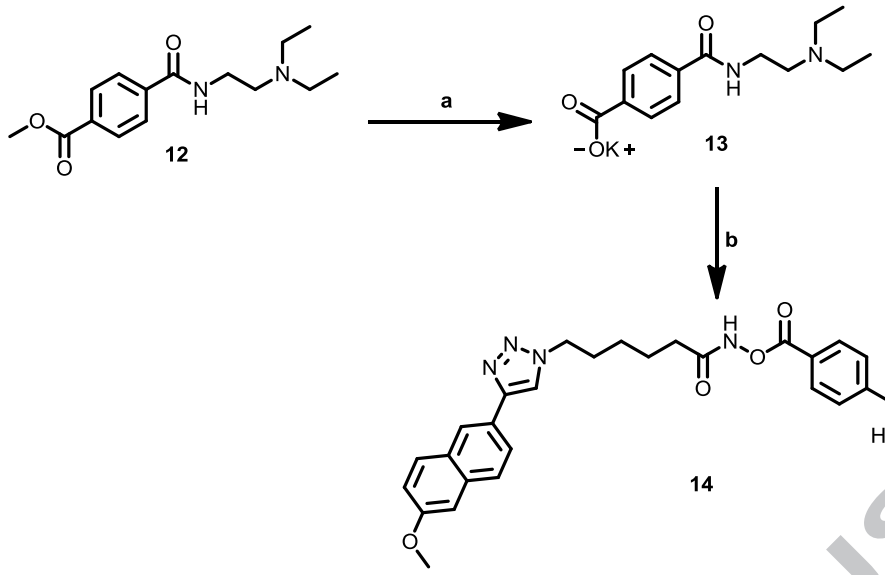


Fig. 3. Proposed prodrug release mechanism

Potassium 4-((2-(diethylamino) ethyl) carbamoyl) benzoate (**13)** used in the synthesis of the prodrug was made as described previously[5c]. Coupling of compound **13** to **7u** using TSTU gave the desired prodrug **14** (Scheme 2).



Scheme 2: Synthesis of releasable BZA-based HDACi. a) KOH, MeOH, rt, overnight; b) Compound **7u**, TSTU, DIPEA, THF/DMF, rt, overnight.

While we do not anticipate any appreciable inhibition of HDAC (because the ZBG is masked), compound **14**, when profiled against HDAC isoforms 1, 6 and 8 at 5 μM, showed 100 percent inhibition against HDAC1, 99 percent inhibition against HDAC6, and 52 percent inhibition against HDAC8. A similar observation was previously reported for some of the compounds made by Miller *et al.* in their patent describing prodrugs of SAHA-like molecules.[19b]

Compounds **7u** and **14** were tested in melanoma cell lines, A375 and B16F10, as shown in Table 3. In B16F10, the prodrug **14** is slightly more potent than compound **10a** and about half as potent as compound **7u**. Similarly, compound **10a** is about half as potent as compound **7u** and equipotent to prodrug **14** in A375 cell line. When dosed at concentration just below IC_{50} (25 μM), prodrug **14** showed the same level of potency as compound **10a**. The advantage to having the prodrug becomes more pronounced at 50 μM, where prodrug **14** becomes much more potent than compound **10a** (Fig. 4). This data implies that there may be an advantage to using the

benzamide template to design prodrugs rather than having it "permanently" incorporated into the design of compounds targeted towards melanin-producing melanoma cells.

Table 3: Anti-proliferation study of prodrug in cancer cell lines.

Compound	**IC_{50} (µM) ***		
	B16F10	**A375**	**LNCaP**
14	27.75	25.95	6.46
7u	10.50	12.61	5.10
10a	34.16	23.60	1.20
SAHA	13.44	4.31	1.80

*values indicate average of three independent experiments

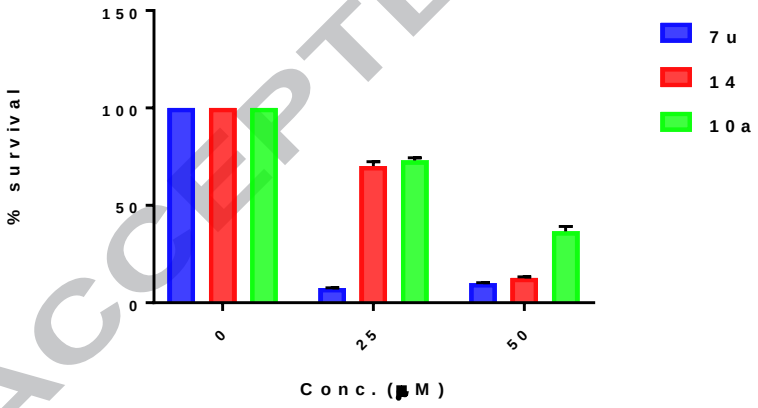


Fig. 4. Comparison of growth inhibitory effects of prodrug **14**, and compounds **7u** and **10a** in B16F10 cell line.

To gain further insight into the cellular accessibility of the synthesized compounds, and confirm that prodrug **14** releases compound **7u** upon getting into the cells, we used western blot to probe for accumulation of acetylated tubulin upon treating B16F10 cells with compounds **10a**, **7u**, **14**, and SAHA. Accumulation of acetylated tubulin in the cytoplasm is a marker of HDAC 6 inhibition in cells.[20] SAHA and compound **7u** as expected showed significant accumulation of acetylated tubulin at 20 μM (Fig. 6). Likewise, compound **10a** gave a similar effect at increasing drug concentrations (5-50 μM). Gratifyingly, prodrug **14** also caused increase in the level of acetylated tubulin in a concentration dependent manner (Fig. 6). This implies that the prodrug is capable of releasing compound **7u** within the cells, validating our prodrug design hypothesis.

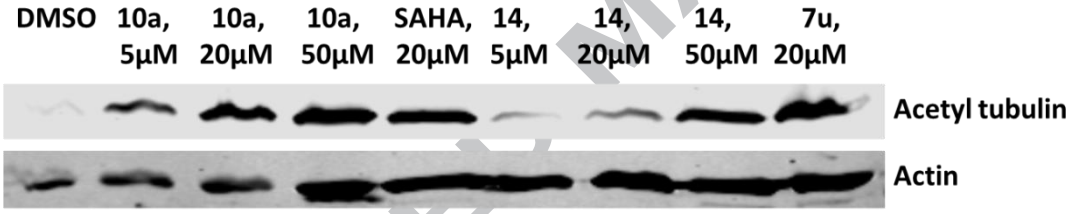


Fig. 6. Western blot analysis confirms that prodrug **14** caused intracellular accumulation of acetylated tubulin.

In conclusion, we reported a series of HDACi **10a-b** and **11a-b** designed to selectively target melanoma cells. These compounds displayed potent HDAC inhibitory activities that are largely dependent on the nature of the cap group and the linker length. Preliminary evaluation of the growth inhibitory effects of these compounds on two melanin-rich melanoma cell lines, B16F10 and A375, showed that they have attenuated antiproliferative activities relative to the untargeted HDACi **7u** and SAHA. Against melanin null prostate cancer cell line LNCaP cell, however, all the compounds showed anticancer activities consistent with their strong HDAC

enzyme inhibitory effects. We believe that the attenuated cytotoxicity of **10a-b** and **11a-b** towards melanoma cell lines may be due to their being bound to melanin in melanoma cells, a scenario which hinders their access to HDACs for therapeutic effect. An alternative prodrug strategy showed some promise as prodrug **14** is slightly more potent than **10a**, a compound having benzamide moiety covalently incorporated into the surface recognition group, in high melanin-producing B16F10 cell line. Further study will be required to optimize benzamide as promoiety to achieve selective delivery of HDACi prodrugs into melanin-producing melanoma cells.

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Supporting Information

Supplementary data associated with this article (^{1}H NMR and ^{13}C NMR spectral information) can be found online.

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Graphical Abstract