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Antibody drug conjugates with hydroxamic acid cargos for histone deacetylase (HDAC) inhibition

The antibody creeping along the streets of the medieval historical center of Siena is an ADC loaded with a hydroxamic acid. Once the target is reached, the conjugate releases its cargo and the hydroxamate drug can hit Histone Deacetylase. In this way it is possible to overcome the poor physicochemical properties of hydroxamic acids.

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Antibody drug conjugates with hydroxamic acid cargos for histone deacetylase (HDAC) inhibition†

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Antitumor hydroxamates SAHA and Dacinostat have been linked to cetuximab and trastuzumab through a non-cleavable linker based on the *p*-mercaptobenzyl alcohol structure. These antibody drug conjugates (ADCs) were able to inhibit HDAC in several tumour cell lines. The cetuximab based ADCs block human lung adenocarcinoma cell proliferation, demonstrating that bioconjugation with antibodies is a suitable approach for targeted therapy based on hydroxamic acid-containing drugs. This work also shows that ADC-based delivery might be used to overcome the classical pharmacokinetic problems of hydroxamic acids.

Hydroxamic acids are a peculiar type of organic molecules featuring a large variety of biological activities. They are potent enzyme inhibitors binding lipoxygenase, hydroxylase, matrix-metalloproteinase, histone deacetylase, carbonic anhydrase, ribonucleotide reductase and several other enzyme families.[1] Besides this wide range of inhibition activities, hydroxamic acids have found therapeutic applications in cancer,[2] cardiovascular diseases, including hypertension,[3] tuberculosis,[4] HIV infection,[5] and Alzheimer's disease.[6] Most of their biological activity is related to the complexation of metals present in enzymes through interaction with *N*-hydroxy and carbonyl groups.[7] They are excellent bidentate ligands for metals such as iron, nickel and zinc. However, translation of this exceptional potential to real drugs has been highly limited by several drawbacks such as mutagenicity, poor pharmacokinetics and several off-target interactions, resulting in ventricular repolarization impairment, thrombocytopenia, gastrointestinal toxicity and several other adverse side effects.[8]

Hydroxamic acids are privileged structures to block histone deacetylase (HDAC) enzymes, and HDAC inhibitors (HDACis) have emerged as one of the most likely classes of multifunctional anticancer drugs.[9] Cell cycle arrest, apoptosis, angiogenesis regulation, activation of tumour suppressor genes and oncogene suppression are some of the down-stream effects observed upon pharmacological treatment with HDACis.[3]

Although acting on the pharmacophore shape through the HDACis structure modification might improve the target selectivity,[10] an attractive alternative would be redirecting the inhibitor to a suitable target using selective delivery. Few examples of hydroxamic acid pro-drug delivery systems have been described to date.[11]

One of the most efficient methods for drug delivery is the antibody–drug conjugate (ADC) strategy, where the antibody drives the drug inside the cell expressing the antibody receptor.[12] Once internalized, the antibody–drug linker is cleaved, and the drug is released inside the malignant cells.[13] The FDA has approved half a dozen ADCs, all of them carrying highly cytotoxic payloads like vedotin, emtansine and ozogamicin, and many others are under clinical trials.[14] It is worth noting that trastuzumab emtansine (T-DM1, Kadcyla) is the only product approved for the treatment of solid tumours.

The current linker technology allows charging antibodies mostly with drugs carrying amines, alcohols or phenols through carbamates or carbonates, while the bioconjugation of drugs with other functional groups is still an undeveloped issue. It is not surprising that the only example of hydroxamic acid linked to antibodies deals with bifunctional chelates bearing hydroxamate arms for the radiometal labeling of monoclonal antibodies.[15]

Following our long-standing interest in HDAC inhibition,[16] we report here the first example of ADC cargos carrying the FDA approved Vorinostat/SAHA (**1**) and Dacinostat/NVP-LAQ824 (**4**),[17] a highly potent HDAC pan-inhibitor with IC_{50} = 32 nM (Scheme 1). These products were conjugated with cetuximab (Ctx) or trastuzumab (Trast), and a preliminary study on their biological activity confirmed the efficient targeting of HDACs within the tumour cells.

Based on our experience with bioconjugation,[18] we started to explore the possibility to link SAHA with mAbs through non-cleavable linkers. Many non-cleavable linkers are based

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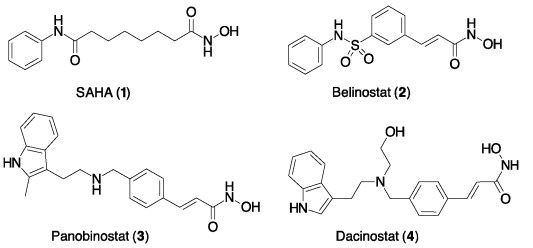
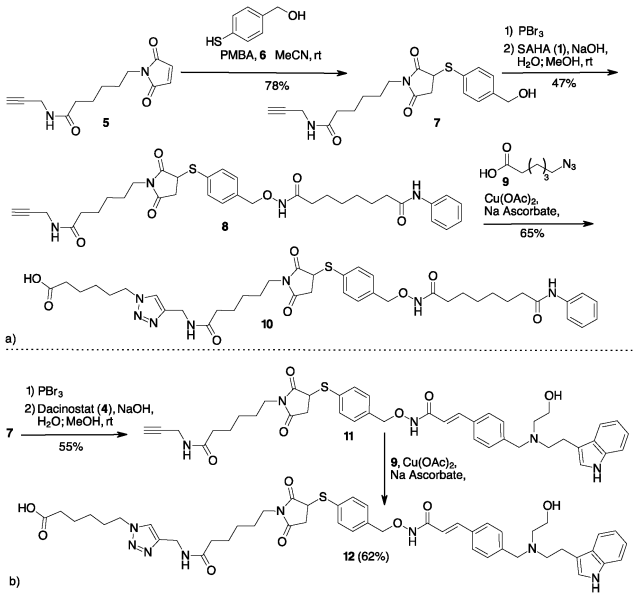


Scheme 1 Hydroxamic acids as power HDAC inhibitors. Compounds **1–3** have been approved by the FDA.

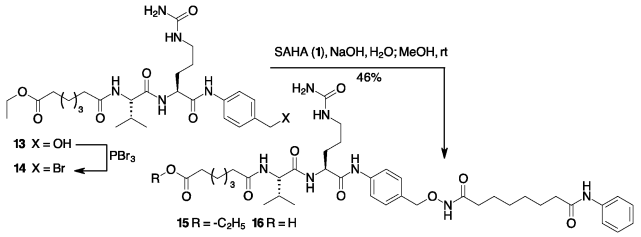
on 3-thiol–maleimide conjugates. During the lysosomal ADC metabolism, a retro Michael-type reaction or a thiol exchange of thioether succinimide occurs in response to thiol-containing environments, enabling drug release.[19] Compound **10** (Scheme 2a) was designed as a potential cargo for antibody conjugation through the amidation of antibody lysines, *via* *N*-hydroxysuccinimide (NHS) activation. Thus, Michael acceptor succinimide **5** was reacted with freshly prepared *p*-mercaptobenzyl alcohol (PMBA, **6**) with the formation of the thioether–maleimide derivative **7**. Alkylation of **7** through the corresponding bromide was accomplished with SAHA in the presence of NaOH, resulting in compound **8** in acceptable yields.

Compound **8** was subject to a Cu catalysed Huisgen reaction with 6-azidohexanoic acid **9** in the presence of Cu(II) acetate and sodium ascorbate in DMF/water, providing the desired compound **10** in 65% yield (Scheme 2a). A similar approach permitted to link Dacinostat (**4**) that was transformed into product **11** with 55% overall yield starting from **7** (Scheme 2b). Cu catalyzed Huisgen cycloaddition furnished **12** in 62% yield.

The synthesis of a Cathepsin B cleavable linker **16** started with the amide **13** composed by dipeptide Fmoc-Val-Cit-OH and *p*-amino benzyl alcohol (PABA) (Scheme 3). Compound **13** was converted into the corresponding bromide **14** with PBr_3.[18]



Scheme 2 (a) Preparation of SAHA containing cargo for lysine bioconjugation. (b) Preparation of Dacinostat containing cargo for lysine bioconjugation.



Scheme 3 Preparation of Cathepsin B sensitive cargo containing SAHA.

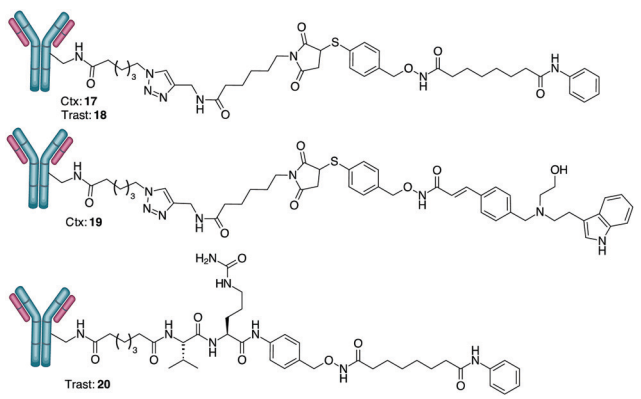
The final introduction of SAHA proceeded in aqueous NaOH to afford product **15**. Compounds **10**, **12** and **15** were stable in PBS and in the human plasma at 37 °C for 24 h (see the ESI,† page 8).

Cargos **10**, **12** and **16** (obtained through hydrolysis of **15** with LiOH) were transformed into the corresponding NHS-esters (ESI,† page 7) and reacted with Ctx, a monoclonal antibody (mAb) specific for the epidermal growth factor receptor (EGFR), and Trast, an mAb targeting the ErbB2 receptor. Conjugation through the stable amide bond afforded ADCs **17–20** (Scheme 4).

Purification of **17–20** was carried out by dialysis, and the drug antibody ratio (DAR) was determined by MALDI analysis. The data show an acceptable degree of homogeneity for ADCs linked through lysine to intact antibodies (Fig. S1–S4, ESI†). The binding of the new ADCs (**17–20**) to EGFR or ErbB2 receptors on tumour cells was assessed by flow cytometry (FACS analysis) on A549 (human lung carcinoma), SKBR3 (human breast carcinoma) and Capan-1 (human pancreas carcinoma) cell lines, expressing different levels of receptors. All ADCs interact with their specific receptor target with a potency comparable to the unconjugated mAbs (Fig. S5, ESI†).

Investigation of ADC internalization was then carried out on the same cell lines by high content screening (HCS) imaging analysis.

Results show that all the ADCs are internalized after binding to EGFR or ErbB2 receptors depending on the target receptor expression level. Internalization was equal with respect to the



Scheme 4 Conjugation of hydroxamates with cetuximab (Ctx) and trastuzumab (Trast). DAR determined by MALDI analysis: **17** DAR = 4 (±0.5); **18** DAR = 5 (±0.5); **19** DAR 3 (±0.5); **20** DAR 6 (±0.5) (average of three experiments).

unconjugated Ctx or Trast, resulting in accumulation within the internal vesicles of the multivesicular bodies and subsequent translocation to the lysosomal compartment (Fig. S6, ESI†).

Release of hydroxamic acids **1** and **4** occurred upon treatment of ADCs **17–20** with human hepatic microsomes. After 72 h of incubation at 37 °C, quantitative HPLC/HRMS analysis showed the presence of peaks at *m*/*z* 264.1472 and 349.1792, respectively, attributed exclusively to compounds **1** and **4** in concentrations corresponding to more than 70% of release of the payload present in the starting ADC.[20]

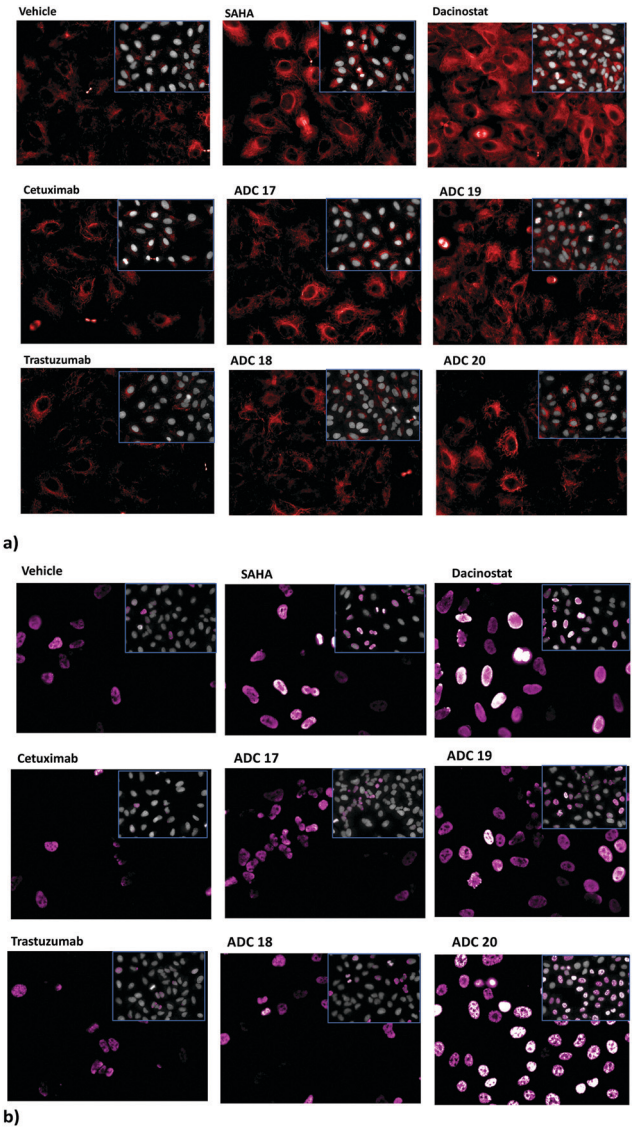


Fig. 1 Effect of SAHA, Dacinostat, Cet and Trast, compared to ADCs **17–20**, on the acetylation of α-tubulin (a) and histone H3 (b) in A549 cells. Cells cultivated for 3 h with reference drugs (100 nM) or antibodies (5 μg mL^{-1}) and stained with anti-acetylated-α-tubulin (red) or anti-acetylated-H3 histone (pink). Insets show the Draq5 dye-stained nucleus and cytoplasm. Fluorescence imaging by HCS Operetta. Data not normalized. Each image is representative of at least 5 fields of duplicate wells. Magnification 60×.

Inhibition of the HDAC activity was determined through HCS-imaging analysis, monitoring the levels of acetylated histone H3 and acetylated α-tubulin in A549 (human lung adenocarcinoma) cells, as the indicators of specific inhibition of nuclear class I HDACs and of cytoplasmic HDAC6, respectively (Fig. 1). Results showed that ADCs **17**, **19** and **20** induced a relevant increase in the acetylation level of α-tubulin and histone H3, as a consequence of direct enzymatic inhibition, while no significant effect was noted in cells treated with Ctx or Trast alone (Fig. 1). Neither cetuximab nor trastuzumab is endowed with HDAC inhibitor activity. So, an increase in the acetylation degree of α-tubulin- or histone H3 is not expected in the cells treated with these drugs. In fact, the extent of acetylation observed in A549 cells following treatment with the above drugs was observed to be similar or apparently a little lower (depending on the fields observed) with respect to the basal level of untreated cells (as shown in Fig. 1). ADC **18** induced an increased acetylation of both α-tubulin and histone H3, although with lower potency with respect to the others. In these cells, the cetuximab-based ADCs **17** and **19** would seem to act with higher potency, probably due to the highest expression of EGFR as compared to ERbB2 receptors on the cell surface. Increased acetylation of histone H3 and α-tubulin was detected through western blot analysis of the total protein lysate. The results of the densitometric analysis of the specific band intensity, after normalization to β-actin signals, showed that the acetylation enhanced upon treatment of tumour cells with ADCs as compared to the vehicle-treated cells and cells treated with Ctx and Trast alone (Fig. S7, ESI†). These results demonstrate that the ADCs are efficiently internalized by the tumour cells releasing the hydroxamic acids required for epigenetic modulation. Similar results were observed on other human tumor (pancreas, melanoma, and breast) cell lines (data not shown).

The anti-proliferative activity of the conjugates **17** and **19** was evaluated in A549 cells upon the treatment, for 6 days, with several doses of ADCs and with equivalent doses of Ctx compared to the unconjugated SAHA and Dacinostat (Fig. 2). ADC **19** inhibited cell proliferation very efficiently in a dose-dependent manner and with quite a similar potency (IC_{50} = 90 ± 3.4 nM) to

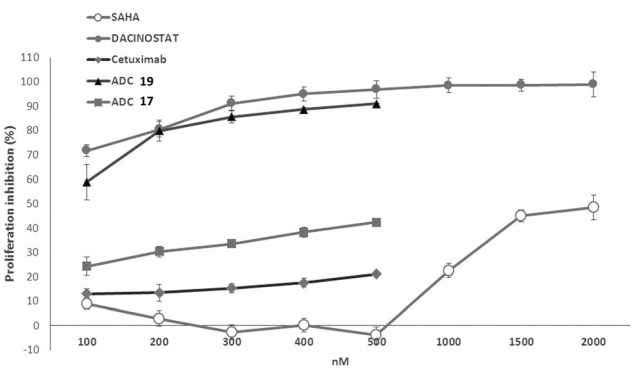


Fig. 2 Antiproliferative activity of ADCs **17** and **19** on A549 cells upon 6 days of treatment. Data are the mean (±SD) of percentage inhibition (n = 5).

that of the reference Dacinostat (IC_{50} = 33 ± 1.7 nM).[21] ADC **17** (IC_{50} = 1440 ± 17 nM) was less efficacious than ADC **19**.[22] Interestingly, ADC **17** retains a certain percentage of activity in a range of concentrations (100–500 nm) in which SAHA resulted utterly inactive.

In conclusion, we have developed a new class of ADCs for the targeted delivery of hydroxamic acids. The new linkers based on PMBA can release the hydroxamic acid after metabolic degradation demonstrating that bioconjugation with mAbs can be employed to develop more effective targeted therapy based on hydroxamate HDACis.[23]

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Conflicts of interest

There are no conflicts to declare.

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