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Research paper

Design of pH responsive clickable prodrugs applied to histone deacetylase inhibitors: A new strategy for anticancer therapy

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ABSTRACT

The aim of this study was to develop clickable prodrugs bearing a tunable pH responsive linker designed for acidic pH-mediated release of histone deacetylase inhibitors. HDACi are an important class of molecules belonging to the epigenetic modulators used for innovative cancer strategies. The behavior of these prodrugs was determined by a bioluminescence resonance energy transfer assay in living tumor cells. This work demonstrated that this innovative type of clickable prodrugs entered cancer cells and showed restored anti proliferative properties attributed to the effective release of the HDAC inhibitors. A correlation between kinetic studies, dose responses, and biological activities was obtained, making such clickable prodrugs good candidates for new strategies in epigenetic-oriented anticancer therapies.

1. Introduction

Epigenetics has emerged in recent years as an important mechanism for the regulation of gene expression through post-translational modifications (PTMs) on the chromatin [1] with applications in various human diseases (cancer, neurodegenerative and metabolic disorders, hepatitis, and others). In eukaryotic cells, the minimal unit of the chromatin is the nucleosome, built from the assembly of eight histones (two copies of H2A, H2B, H3, and H4) and DNA [2]. Each nucleosome is separated by a fifth histone: H1. This DNA packaging is responsible for the regulation of gene transcription. PTMs are reversible chemical reactions occurring on DNA and on histones. While the major reaction on DNA is the methylation of CpG islands [3], several modifications are known for histones, mainly on the lysine residues found in the terminal tails of histones H3 and H4. The combination of these PTMs can be exclusive or complementary and is recognized by transcription factors in a selective way, leading to the repression or stimulation of gene transcription and defining the so-called histone code [4]. Epigenetics plays nowadays an important role in cancer researches [5]. The most studied histone PTM is lysine acetylation, with histone acetyl transferases (HAT) [6] responsible for the N-ε acetylation of lysine while histone deacetylases (HDAC) [7] remove this acetyl group (Fig. 1). This acetylation equilibrium is part of the mechanisms controlling gene expression and is recognized by bromodomain-containing transcription factors [8].

HDACs appeared over expressed in several cancer cell lines resulting in abnormal hypoacetylation of histones, favoring in particular tumor suppressor gene silencing. Thus, inhibition of HDACs appeared to be an innovative anticancer strategy and a large number of studies demonstrated the potential of HDACi on different cancer cell lines and on animal models of tumors [9]. HDAC inhibitors (HDACi) entered clinical trials [10] and two drugs were approved to treat cutaneous T-cell lymphoma (CTCL): Vorinostat (Zolinza®) = Suberoyl Anilide Hydroxamic Acid (SAHA) and Romidepsin (Istodax®) (Fig. 1). SAHA is a non-specific HDACi while Romidepsin is considered to be a natural disulfide prodrug cleaved in cells by glutathione [11]. Entinostat (MS-275) and CI-994 illustrate the benzamide group (Fig. 1). Valproic acid (VPA), a carboxylic acid approved to treat epilepsy, is a millimolar HDACi, often used due to its well-known clinical profile, whereas HDACi have showed high efficacy on hematological diseases such as myelodysplastic syndromes (MDS) and CTCL. Their effects on solid tumors

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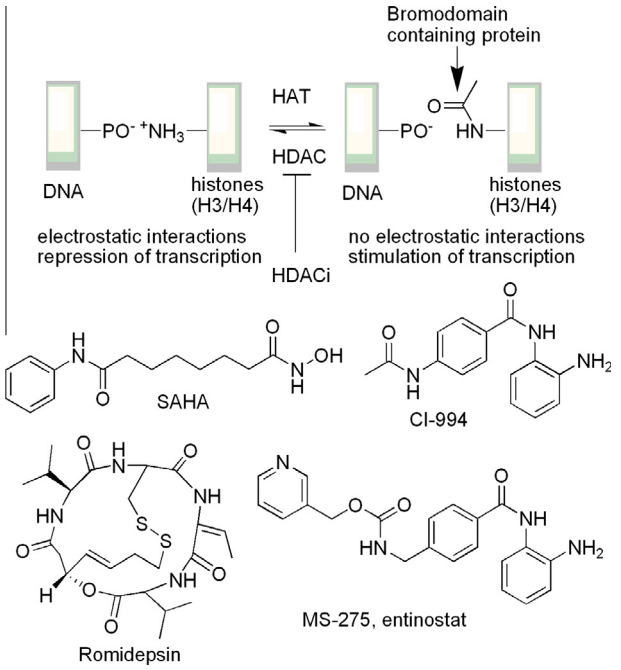
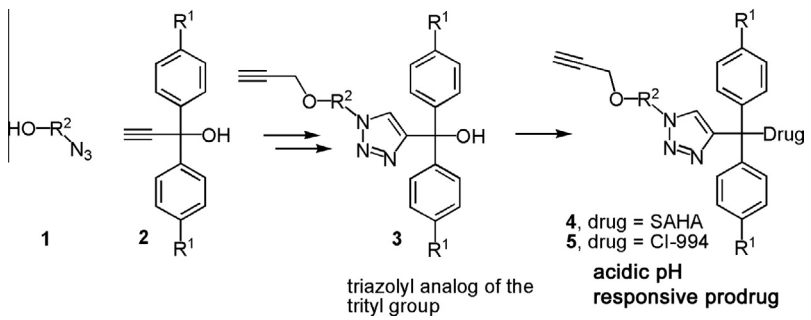


Fig. 1. Top: Principle of reversible acetylation of histones by HAT/HDAC and its recognition by bromodomain-containing proteins, with therapeutically positioned HDAC inhibitors. Bottom: examples of HDAC inhibitors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were limited due to their hematological toxicity, mainly thrombopenia, and cardiotoxicity which limit the doses administrated [10]. Another main limitation is their rapid clearance. SAHA is metabolized in less than 1 h by glucuronidation [12] and by beta-oxidation, like VPA [13], and some resistance are known for hydroxamic and carboxylic acids [14]. Romidepsin is metabolized as glutathione conjugate [15] or by the action of P450 enzymes [16]. Resistance has also been demonstrated for Romidepsin [17]. Benzamides are more stable with 1 or 2 days half-life.

All these constrains are responsible for the low level of compounds reaching the malignant tissues and make HDACi good candidates for ligation to prodrugs able to release them slowly over time only in cancer cells in order to reduce side effects and metabolism. An important strategy developed for efficient cancer cell internalization of bioactive molecules relies on the endocytosis pathway [18]. To our knowledge, this delivering strategy has never been applied to HDACi and appeared of high interest to circumvent the clinical limitations of this class of compounds. Drug delivery based on endocytosis-mediated internalization exploits the initial formation of endosomes with moderately acidic pH (pH = 6) leading after maturation to lysosomes with a more acidic pH (pH = 4–5). Thus, pH responsive carriers can be used to transport and release compounds during endocytosis with typical pH responsive groups like trityl [19], benzaldehyde [20], and hydrazone [21] depending on the linked molecules. For Doxorubicin, a hydrazone derivative on the side chain carbonyl group and a carbamate group on the amino sugar were described as prodrugs for acidic pH-mediated release. The hydrazone strategy gave better results [22] showing that simple carbamate are not useful in this case for acidic release. Trytil and benzaldehyde groups were used for nucleosides derivatives. In contrast, we demonstrated in a recent contribution to this field that the azide–alkyne click chemistry approach leading to triazole rings can be used as a general ligation strategy producing at the same time pH responsive prodrugs. Carbamate derivatives and analogs of the trityl group were prepared by the same synthetic strategy all retaining acidic pH sensitivity [23]. These results indicated that our generic, flexible, and readily synthesized tunable pH responsive groups are valuable alternative to prepare prodrugs of the various functional groups found in bioactive molecules. Another point of interest is the protonation of triazole rings, exploited for the preparation of membranes for proton transfer [24a]. This property could be useful for vesicle escape during endocytosis. It has been demonstrated that polyimidazoles used as vector for DNA (so-called polyplexes [24b]) can escape the endocytosis pathway at the endosome level to avoid later DNA degradation in the more acidic lysosomes. This escape is the result of modification in proton gradient in the endosomes due to imidazole protonation. Protonation of the triazole could lead to the same effect.

We aimed applying these results for the preparation of pH responsive diaryltriazolyl-based prodrugs of the important therapeutic class of HDACi. Our preliminary results on anilines prompted us to select CI-994, a HDACi member of the benzamide group previously used in combination trials and related to Entinostat, the latter currently in phase I/II clinical trials. SAHA was also chosen for its clinical importance and rapid clearance. SAHA has been involved in 2012 in several phase I/II clinical trials in combination with other anticancer compounds and to fight various cancer types. The possibility to obtained controlled release and possibly for different molecules from the same pH responsive structure could thus also have high interest in differentiated release. We envisioned the covalent linking of these two molecules in a protecting prodrug way through their respective hydroxamic acid and benzamide functions, both being prone to metabolic activities. Having also in mind to develop a prodrug system that could be further functionalized, we modified our initial pH responsive system, introducing a second alkyne–azide click chemistry site. Our double click synthetic strategy involves the key monopropargylated ether **3** (Scheme 1), where a triazol ring is first formed to produce the pH responsive part while an alkyne ether part will serve for future modifications by a second click reaction. In particular, this second click site could be used for further grafting onto drug delivery system (DDS), provided this DDS bears an



Scheme 1. Design of acidic pH responsive releasing systems for differentiated "double click" strategy.

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azide group or to add detectable properties for in vivo imaging [25]. The conversion of the alcohols **8** to ethers **3** is expected to increase the hydrophobic character of the molecules, but on the other hand, this strategy also avoids the protection/deprotection of the terminal primary hydroxy group prior to drug insertion that could make the approach more complex. Blocking this primary hydroxyl group for drug insertion is necessary as we demonstrated previously that primary alcohols can be used as drugs with our system [23c]. Thus, preparing prodrugs from alcohols **8a–c** could give intermolecular attack of these alcohols to yield undesired polymers instead of the expected alkyne-free prodrugs. A spacer R^2, selected to be biologically inert and to potentially improve the water solubility, was inserted between the two click sites to account for the bulkiness of the pH responsive part. Spacers are usually necessary to make the reactive site of prodrugs easily accessible especially when enzymes are involved in the releasing mechanism.

We report in this work our synthetic findings to obtain a differentiated double azide–alkyne click chemistry on the same structure, its application to the preparation of new clickable pH responsive HDACi prodrugs and their biological evaluations (Scheme 1, drug = HDAC inhibitor (HDACi)). Three versions were prepared (various R^1 groups) for each HDACi and were evaluated in cancer cells for their ability to release the inhibitors with restoration of HDAC inhibition measured by a BRET assay. The final impact on cancer cell viability was also measured.

2. Materials and methods

2.1. Materials

All solvents were dried before use when required with classical methods. Distilled water was used for cycloaddition under Sharpless conditions. 250 μm silica gel plates were used for TLC analysis. All reactions were conducted under nitrogen, and concentrations were performed under reduced pressure using a rotary evaporator. Extracted organic layers were dried with $MgSO_4$ and the solvents removed under reduced pressure. Purifications were made by column chromatography with silica gel (35–70 μm silica) or using Combi Flash apparatus for compound **4** and **5**. DCM = CH_2Cl_2, THF = tetrahydrofuran, EA = ethyl acetate, PE = petroleum ether 35–60°, ACN = acetonitrile. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra are given in ppm as referenced to TMS (tetramethylsilane) as internal standard. ^1H NMR coupling constants are reported in Hertz and refer to apparent multiplicities and not true coupling constants. Data are reported as follows: chemical shift, multiplicity (s = singlet, br s = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublets of doublets, etc.), integration, and coupling constant. HRMS were performed in the Centre Régional de Mesures Physiques de l'Ouest, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes, France.

2.2. Methods

2.2.1. Typical synthesis of compound *4*

N-phenyl-N′-[[1-[2-[2-[2-(2-prop-2-ynoxyethoxy) ethoxy] ethoxy] ethyl] triazol-4-yl]-bis(phenyl) methoxy] octanediamide **4a**. *Method A*: To a solution of **3a** (0.20 g, 0.43 mmol) in DCM (5 mL) was added HCl (2 M in Et_2O, 0.3 mL, 0.86 mmol). After 2 h reflux, the solution was co-evaporated with toluene to give the crude chloride **10a** as oil. To a solution of SAHA (0.340 g, 0.129 mmol) and dry NEt_3 (0.24 mL, 1.72 mmol) in ACN (5 mL) was added the crude chloride **10a** dissolved in a minimum of ACN at ambient temperature. The resulting solution was stirred overnight at room temperature, then filtered, and washed with ACN to recover unreacted SAHA as a solid. The concentrated filtrate was purified by Combi Flash (DCM/EtOH/Et_3N 94:5:1) to give **4a** as a colorless oil (0.052 g, 0.073 mmol, 17%). The unreacted alcohol **3a** is also recovered. *Method B*: To a solution of **3a** (0.11 g, 0.25 mmol, 1 eq.) in dry toluene under nitrogen atmosphere was added AcCl (0.09 mL, 1.25 mmol, 5 eq.). After 2 h reflux, the solution was evaporated to give the crude chloride **10a** as oil. The addition of SAHA, work-up, and purification was performed as for method A. Compound **4a** was obtained in better yields with method B (0.075 g, 0.107 mmol, 42%). The unreacted alcohol **3a** (0.010 g, 10%) and SAHA (0.124 g, 36%)) were also recovered.

TLC MeOH/CH_2Cl_2/Et_3N 5:94:1 Rf = 0.33; **^1H NMR** (400 MHz, Acetone-D_6): δ = 9.75 (s, 1H), 9.08 (s, 1H), 7.69–7.65 (m, 3H), 7.46–7.41 (m, 4H), 7.34–7.25 (m, 8H), 7.04–7.00 (m, 1H), 4.57 (t, 2H, J = 5.1 Hz), 4.16 (d, 2H, J = 2.4 Hz), 3.88 (t, 2H, J = 5.2 Hz), 3.62–3.55 (m, 6H), 3.53–3.49 (m, 6H), 2.93 (t, 2H, J = 2.4 Hz), 2.31 (t, 2H, J = 7.5 Hz), 1.93 (t, 2H, J = 7.2 Hz), 1.60 (m, 2H), 1.39 (m, 2H), 1.25 (m, 2H), 1.12 (m, 2H); **^{13}C NMR** (100 MHz, Acetone-D_6): δ = 26.0, 33.7, 37.7., 50.8, 58.6, 69.8, 70.1, 70.9, 71.1, 71.16, 71.2, 71.22, 75.8, 80.9, 115.7, 119.9, 123.8, 126.7, 128.5, 128.6, 129.2, 129.4, 133.6, 140.8, 161,0, 172,0; **HRESI-MS**: calcd. for $[M+Na]^+$ ($C_{40}H_{49}N_5O_7Na$): 734.35242, found: 734.3524.

2.2.2. Typical synthesis of compound *5*

N-(2-((1-(3,6,9,12-tetraoxapentadec-14-ynyl)-1H-1,2,3-triazol-4-yl) diphenylmethyl amino) phenyl)-4-acetamidobenzamide **5a**. Chloride **10a** was prepared as above and dissolved in minimum ACN (2–3 mL), and the resulting solution was added to a solution of CI-994 (0.231 g, 0.90 mmol) in ACN (5 mL) containing Et_3N (0.24 mL, 1.72 mmol). The solution was stirred 12 h at ambient temperature and concentrated, and the crude material was dissolved in acetone from which the unreacted CI-994 was precipitated and filtered off for recycling. The filtrate was purified (Combi Flash DCM/MeOH/Et_3N 97:2:1) to give **5a** as an oil (0.142 mg, 0.17 mmol, 39%). The unreacted alcohol **3a** was also recovered. **^1H NMR** (400 MHz, Acetone-D_6): δ = 9.44 (bs, 2H), 8.02 (d, 2H, J = 8.6 Hz), 7.82 (s, 1H), 7.77 (d, 2H, J = 8.7 Hz), 7.65 (m, 4H), 7.24 (m, 5H), 7.18 (m, 2H), 6.70 (dt, 1H, J = 1.6 Hz, J = 8.1 Hz), 6.61 (dt, 1H, J = 1.3 Hz, J = 7.5 Hz), 6.17 (dd, 1H, J = 1.1 Hz, J = 8.2 Hz), 6.12 (s, 1H), 4.50 (t, 2H, J = 5.2 Hz), 4.14 (d, 2H, J = 2.4 Hz), 3.79 (t, 2H, J = 4.9 Hz), 3.59 (m, 2H), 3.55 (m, 2H), 3.48 (m, 4H), 3.43 (m, 4H), 2.92 (t, 1H, J = 2.4 Hz), 2.12 (s, 3H); **^{13}C NMR** (100 MHz, Acetone-D_6): δ = 169.3, 152.9, 146.5, 143.5, 141.9, 129.9, 129.5, 128.9, 128.8, 127.7, 127.5, 126.8, 126.5, 125.9, 119.2, 118.6, 118.1, 81.0, 75.8, 71.21, 71.17, 71.0, 70.2, 69.8, 65.7, 58.6, 50.9, 24.4, 22.9, 15.2; **HRESI-MS**: calcd. for $[M+Na]^+$ ($C_{41}H_{44}N_6O_6Na$): 739.32145, found: 739.3215.

2.2.3. Determination of hydrolysis rates of prodrugs in mildly acidic pH

The compounds **4a–c** and **5a–c** were submitted to acidic hydrolyzes with buffered solutions. 0.4 mL of a 0.5 mg/mL solution of the compounds in ACN was added to 1.6 mL of buffer under vigorous stirring. At this concentration, no precipitation of the starting compound was observed for any of the derivatives analyzed. HPLC of the starting prodrugs and buffers alone in the mixture of ACN/H_2O used for hydrolysis conditions were used to validate the drug release and reformation of alcohols **3**. No unknown products were formed in these conditions. The acidity is being probably not strong enough for Ritter reaction with ACN [26]. The final solutions tested were thus composed of 20:80 ratio of acetonitrile/buffer with pH values in the range 4.3, 5.0, 6.0, and 7.3. The clear solution was then injected at different times directly with an automated High Pressure Liquid Chromatography (HPLC) apparatus equipped with a DAD Hitachi L-2455 (detection range: 200–400 nm), an autosampler Hitachi L-2200, a pump Hitachi L-2130, and a reversed phase HPLC column Lichrocart 150-4,6 purospher STAR (Supplementary information for eluting systems). The peaks corresponding to the starting material and the released alcohols were integrated and

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compared to determine the hydrolysis kinetics. All peak attributions were confirmed by comparison of the UV spectra from 200 to 400 nm of pure material. A co-injection with the starting material at the end of the hydrolysis was also made when necessary to detect any deviation in retention times during the experiments and to confirm the disappearance of the starting prodrugs.

2.2.4. Drugs

For biological tests as control, SAHA was purchased from R&D chemicals and CI-994 was obtained from Sigma–Aldrich. SAHA and CI-994 used for prodrugs synthesis were prepared according to literature procedures. All drugs and prodrugs tested were dissolved in DMSO at a stock concentration of 10 mM.

2.2.5. Cell culture

The pleural mesothelial cell line, MeT-5A, was obtained from American Type Culture Collection (ATCC). The mesothelioma, Meso13 and Meso34, and adenocarcinoma (ADCA), ADCA 153 and ADCA 72, cell lines were established from the pleural fluids of mesothelioma or lung ADCA patients, respectively [27]. All cell lines were maintained in RPMI medium (Invitrogen) supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 0.1 mg/mL Streptomycine, and 10% heat inactivated fetal calf serum (FCS) (Eurobio).

2.2.6. Transfections

MeT-5A cells were seeded at a density of 2.5×10^5 cells per 35 mm dish. Transient transfections were performed 1 day later using Attractene (Qiagen), according to the manufacturer's protocol. For BRET experiments, MeT-5A cells were transfected with 0.6 μg Rluc-Brd cDNA and 1 μg YFP-fused histone H3 cDNA [28]. One day after transfection, cells were transferred into 96-well microplates (microplate 96 well, white, Berthold Technologies) at a density of 3×10^4 cells per dish in 180 μL of culture medium. Treatments were performed by addition of 20 μL of compounds solutions to obtain 200 μL of final culture medium. The compound concentration used for the 20 μL addition was 10-fold higher than the expected final concentration in the culture medium to account for the dilution effect. The addition was made at different times depending on the experiment. BRET measurements were performed as described below.

2.2.7. BRET measurements

All BRET measurements were performed at room temperature using the Mithras LB 940 microplate analyzer (Berthold Technologies). Cells were pre-incubated for 15 min in PBS in the presence of 2.5 μM coelenterazine, following which light-emission acquisition at 485 and 530 nm was carried out. Plates were measured five times. The BRET signal was expressed in milliBRET units (mBu). The BRET unit has been defined previously as the signal ratio 530/485 nm obtained when the two partners are present, corrected by the ratio 530/485 nm obtained under the same experimental conditions, when only the partner fused to Renilla luciferase is present in the assay [29].

2.2.8. Determination of cell viability

Cell growth of Meso13, Meso34, ADCA153, and ADCA72 was monitored using Uptiblue (Interchim). Reduction of this compound by the cell results in the formation of a fluorescent compound quantified by measuring fluorescence at 595 nm after excitation at 532 nm using a Typhoon apparatus. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well in 180 μL of culture medium. Twenty-four hours later, 20 μL compounds solutions were added for 72 h. The compound concentration used for the 20 μL addition was 10-fold higher than the expected final concentration in the culture medium to account for the dilution effect. Uptiblue (5%, v/v) was then added to the culture medium for 2 h at 37 °C. Fluorescence was quantified by measuring emission at 595 nm after excitation at 532 nm using a Typhoon apparatus.

3. Results and discussion

3.1. Synthesis

A polyethyleneoxide (PEO) chain with 4 units was chosen as spacer R_2 (Scheme 1) to prepare the known compound **1** (Scheme 2) [30]. Ketones **7a–c**, purchased or prepared from chloride **6b** for **7b**, were converted to alkynols **2a–c** and reacted with the azide **1** by copper catalyzed Huisgen' cycloaddition to give alkynols **8a–c** in high yields (Scheme 2). The propargylation of alkynols **8a–c** gave the expected inseparable mixture of monoether **3** and diether **9**. This difficulty was circumvented by a treatment with two equivalents of base and propargylbromide to give exclusively the diether **9**. Treatment with 1 M $KHSO_4$ of the **3**:**9** mixture or the diether **9** alone gave only the desired monoether **3a–c**. The expected sensitivity to acidic pH of the prodrugs was exploited to selectively hydrolyze the ether **9** while the monoether **3** was stable under such conditions. This result also confirmed the application of such system as pH responsive prodrugs for alcohols. The syntheses of the key intermediates **3** were achieved in 4 steps in high yields from diols **8a–c**. Alkynols **3a–c** were then converted to the intermediate chlorides **10a–c** and used directly for substitution by SAHA and CI-994 (Scheme 2). The final linking of the drugs to afford the prodrugs **4** and **5** gave moderate yields due to limited solubility of the drugs in our experimental conditions. Attempts to improve the yields (added DMSO, various bases (various equivalents of NEt_3, collidine, lutidine) and chlorination methods (AcCl/reflux or HCl/Et_2O) gave limited results, but the starting material can be recovered easily. Unreacted SAHA and CI-994 could be recycled by precipitation from the reaction mixture with acetone or acetonitrile in 36–73% and 37%, respectively, while the alkynols **3a–c** were recovered during purification of the filtrate by chromatography in 10%, 38%, and 40%, respectively. This recycling strategy allowed preparing up to 300 mg of compound **5a** and 240 mg of compounds **4b**.

SAHA and CI-994 were found linked to alcohols **3** as shown in Scheme 2. No alternative ligations were detected. In the case of CI-994, only the free NH_2 group is reactive, when compared to the two other amide groups that require more basic conditions for activation. In the case of SAHA, the hydroxyl group is more reactive than the nitrogen in both amide groups (benzamide or hydroxyamide). All compounds **4** and **5** were >95% purity (HPLC Supplementary information) with no free drug content. The stability of prodrugs in neat form and in DMSO solution stored at −20 °C was confirmed by several biological experiments over months.

3.2. Hydrolysis rates

Compounds **4** and **5** were then submitted to buffered solutions at biologically relevant pH to monitor the HDACi release over time [23]. As expected, the more electron donating group on the phenyl rings (R^1 groups) the faster the hydrolysis (Table 1). With the more sensitive compounds having half-life less than minutes the apparent half life was difficult to determine with accuracy due to the experiment schedule. For this reason, half-life is indicated as being below minutes. Compounds **5** were all more sensitive to acidic conditions compared to compounds **4**. This result was expected from our previous studies on alcohols (to be compared to hydroxyamides) and anilines [23d], showing the higher stability of the alcohols [23c]. As a comparison at pH 4.3, compound **4a** was stable while **5a** was rapidly hydrolyzed and compound **4b** was 1200-fold more stable than **5b**. These differences were reduced at pH 6 with

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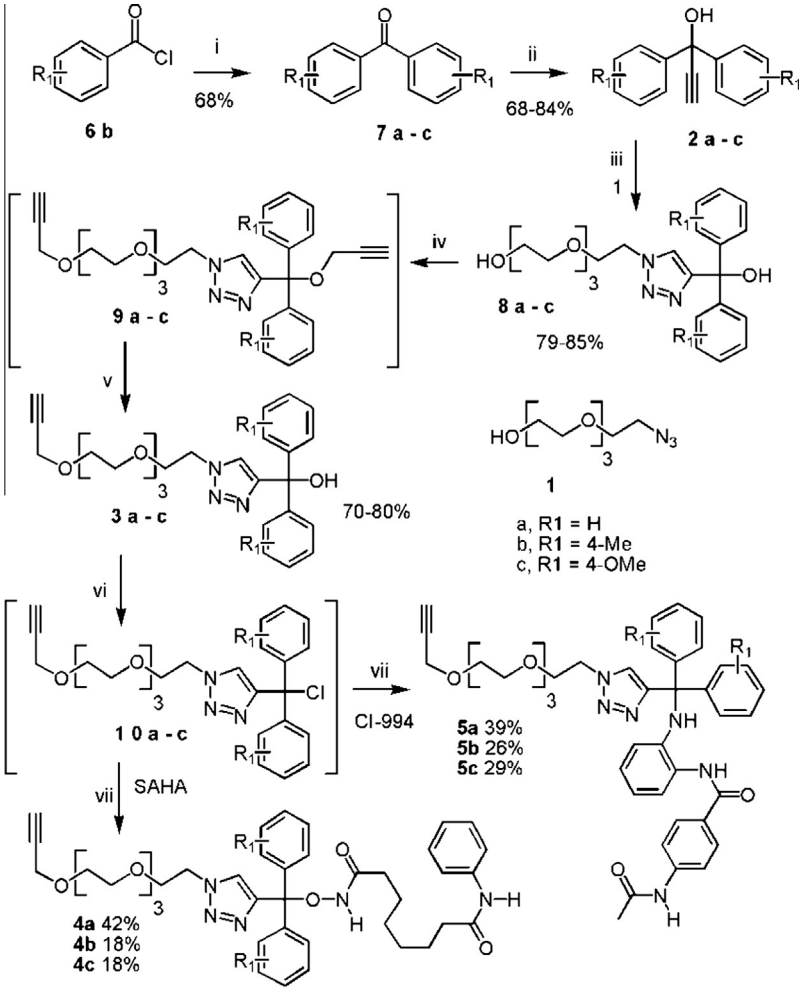

Scheme 2. (i) R^1—Ph, CH_2Cl_2, $AlCl_3$. (ii) a – TMS—C≡C—H, nBuLi, THF; b – KOH, MeOH. (iii) CuI, EtOH:CH_2Cl_2. (iv) THF, NaH 2 eq., Br—CH_2C≡CH 2 eq. (v) $KHSO_4$ 1 M. (vi) HCl in Et_2O, or $SOCl_2$. (vii) NEt_3, ACN.

for instance prodrug **4b** being only 2-fold more stable than **5b**. Both **4c** and **5c** were rapidly hydrolyzed, highlighting a possible susceptibility of the **c** series to nucleophilic attack, a result previously observed with di- or trianisylmethyl chlorides, very sensitive to water hydrolysis [31]. In tumor cells, the pH environment was described as more acidic than in normal tissues while the intracellular pHs for endosomes and lysosomes is around 6 and 5, respectively [24c]. The hydrolysis rate measured with pH 6 buffer indicated that endosomal hydrolysis (and drug escape) should be very slow for compounds **4a, b,** and **5a** and that the process will be faster at the lysosomal level.

For SAHA (Table 1), derivative **4a** (Scheme 2, R^1=Ph) was stable in the pH range selected in agreement with literature data indicating that trityl protected hydroxamic acids were stable in basic conditions and required higher acidity for cleavage. There is no methyl or methoxy substituted trityl hydroxamic acids described to our knowledge in the literature for comparisons purposes. At the opposite compound, **4c** was rapidly hydrolyzed at all tested pHs. The best system for the expected endocytosis-mediated release of SAHA appeared to be the intermediate version **4b**, with a half-life of 20 h at pH 4.3 and with a 4 days half-life at pH 7.3. Despite the apparent limitation at physiological pH, this result is an improvement for SAHA delivery, the free molecule being metabolized in less than 1 h. In the case of CI-994, once again version **5c** was hydrolyzed rapidly at all pHs and the best solution was the derivative **5a** with a 30 min half-life at pH 4.3 and with a 4 days half-life at pH 7.3. For compound **5c,** this result is very different compared to previous studies with aniline prodrugs stable at pH 7.4. This could be due to the electron withdrawing effect of the ortho-amide group, influencing the Ar_2triazolyl C—N bond strength or reactivity.

Table 1
Half-lives for compounds **4a–c** and **5a–c** in buffered conditions.

	pH 4.3	pH 5.0	pH 6	pH 7.3
4a	Stable	Stable	Stable	Stable
4b	20 h	–	80 h (3.3 days)	4 days
4c	30 min	80 min	108 min	34 min
5a	30 min	400 min	48 h	3.6 days
5b	<1 min	12 min	10 min	2 h 30
5c	<1 min	<5 min	<5 min	2 h 30

The lysosomal maturation being less than 1 h and the lysosomes remaining active for hours, we hypothesized that these two candidates **4b** and **5a** should correspond to our needs, as at pHs 5 and 6, they have long intermediate half-lives, considering the application for cell delivery through endocytosis and requirement for stability in blood plasma.

From a mechanistic point of view, these results may also be explained by Scheme 3. After protonation of compounds **4** or **5** on the hetero atom linked to the tertiary carbon, suggested as the more nucleophilic site, the active molecule is released to give the

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Table 2
EC_{50} values for compound-induced BRET signal.

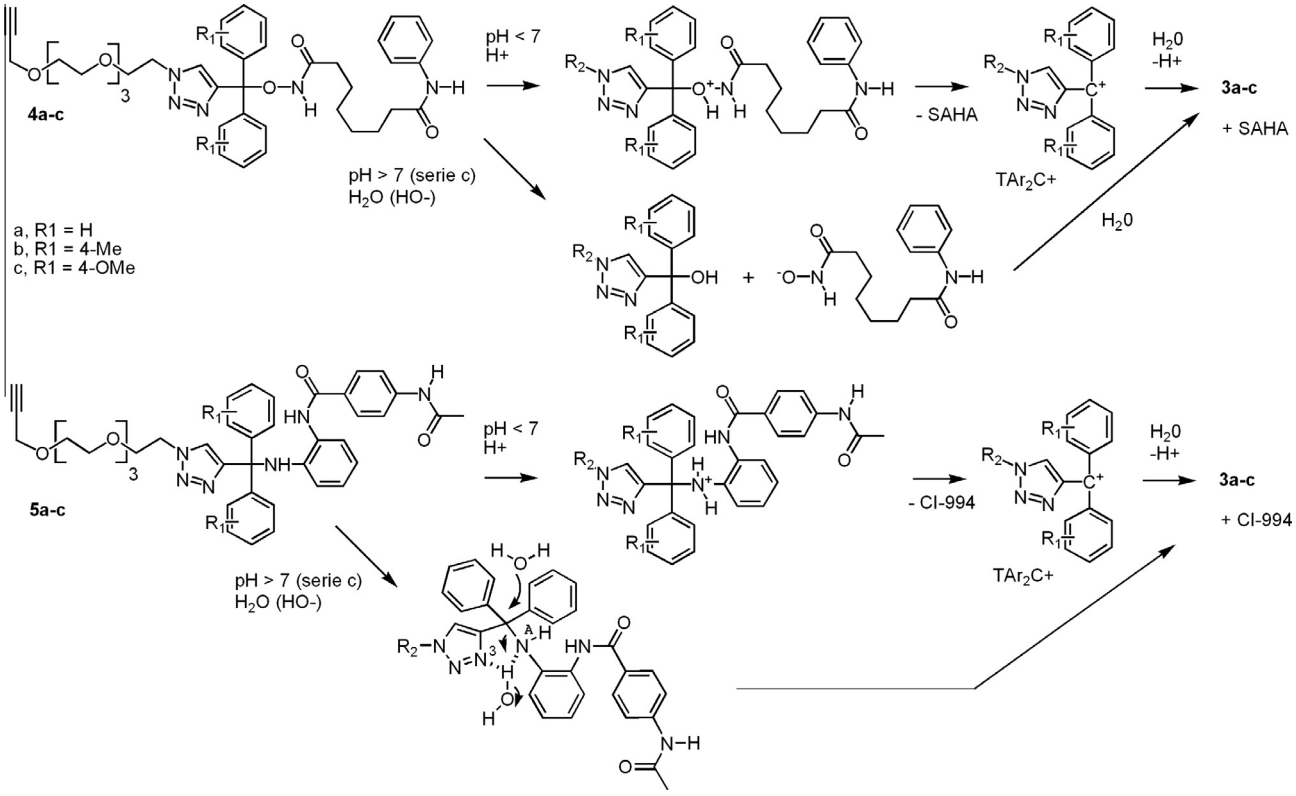
Compounds	EC_{50} (μM)
SAHA	0.42 ± 0.08
4a	7.17 ± 3.19
4b	0.59 ± 0.18
4c	0.75 ± 0.07
CI-994	0.86 ± 0.08
5a	6.20 ± 1.37
5b	0.68 ± 0.12
5c	8.88 ± 3.89

EC_{50} values were determined using GraphPad prism, Prism 5 for Windows, by curve fitting using a sigmoidal dose–response model. Results are the means ± SEM. of three independent experiments.

corresponding carbocation TAr_2C^+, stabilized by the two phenyl groups. This carbocation is finally trapped in a Sn_1 mechanism by water to give alcohols **3** after proton release. If the released molecules are sufficiently nucleophilic, a retro attack of the carbocation will limit the apparent release kinetics. In acidic conditions, this equilibrium between carbocation stability and the nucleophilic character of the active molecule are orienting the reaction. Once protonated, the CI-994 is released and the free aniline can be itself protonated, limiting retro attack and accounting for apparent faster kinetics compared to SAHA. Alternatively, a possible direct water substitution on the protonated prodrug intermediate can be proposed leading to drug release and formation of protonated alcohols **3**. A final deprotonation will give the free alcohols. For series **c,** a mechanism can also be proposed involving the participation of carbonyl groups [32] or the triazole ring favoring local water dissociation. In our previous work, we demonstrated that the N^3 nitrogen atom can be protanated first if the bioactive molecule is linked as a carbamate [23b,d]. It has been postulated that Sn2 reaction are not favored for tertiary alkyl and that alternative Sn1 mechanisms could be involved, with pre-association mechanisms and intimate or solvent ion pairing formation [33]. It can be suggested for CI-994 that the electron attractive effect of the ortho carbonyl group gives a less basic N^A atom, favoring a transition state where a water molecule is interacting with the N^3 atom prior to hydrogen transfer to the N^A nitrogen atom. Alternatively, we showed in a related work that aniline carbamate prodrugs bearing a electron withdrawing group in ortho position react in nucleophilic conditions to yield oxazolidinone by intramolecular cyclization as well as aniline release [34]. Such mechanisms could be involved in the present work for water dissociation assistance. This suggested mechanism can also be applied to SAHA, with the formation of an intermediate anion. This anion can be stable to some extent, favoring solubility or diffusion, but eventually also the re-attack of TAr_2C^+, if present.

3.3. Dose-dependent restoration of HDAC inhibition measured by BRET assay

We previously developed a practical BRET assay to determine the ability of molecules to induce histone H3 acetylations based on HDAC inhibition [28]. This assay allows accelerated biological evaluations of potential HDACi directly in living cells and avoids complex protocols like Western blotting. Briefly, interactions between bromodomain and acetylated histones were exploited to engineer phRluc-C1-BrD and pEYFP C1-histone H3 cDNA transfected in cells. phRluc-C1-BrD cDNA encodes a bromodomain fused to Renilla Luciferase (RLuc) and pEYFP C1-histone H3 cDNA encodes histone H3 fused to Yellow Fluorescent Protein (YFP). An increase in BRET signal reflects an increase in histone H3 acetylation, indicating that free HDACi enter the cells and block the nuclear HDACs activities. In this work, several cancer cell lines were used for comparing the compound activities. The immortalized pleural



Scheme 3. Postulated release mechanisms for compounds **4** and **5** in acidic and moderately basic conditions.

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mesothelial cell line MeT-5A, was obtained from American Type Culture Collection (ATCC), the malignant pleural mesothelioma, Meso13 and Meso34, and adenocarcinoma cell lines (ADCA: ADCA 153 and ADCA 72) were established from the pleural fluids of mesothelioma or lung ADCA patients, respectively [29]. The BRET assay was used to determine dose responses and kinetics of the releasing systems. Compounds **3**, **4a–c,** and **5a–c** were evaluated using our BRET assays and for antiproliferative activities on MPM and ADCA cancer cell lines.

BRET-based dose response experiments were performed first to determine the half maximal effective concentration (EC_{50}) for SAHA, CI-994 and their derivatives **4** and **5** and the released alcohols **3** (Table 2).

Compounds **3** were expected to have no HDAC inhibition as confirmed by Fig. 2 (top right) where no BRET induction was observed. They were also found inactive in our cancer cell lines as shown by Fig. 5 (bottom).

SAHA and prodrugs **4** gave all BRET signals as a sigmoid dose response profile (Fig. 2: SAHA top left, compounds **4** bottom left). This confirmed the expected HDACi activity for SAHA and that prodrugs **4** were able to release SAHA to obtain restored HDAC inhibition. The prodrugs **4** by themselves are supposed to have no activity on HDACs as the HDACi binding group is blocked (hydroxamic acid). Some differences were observed between the three releasing systems **a**, **b** and **c** and the free HDACi. For compounds **4c** with the HDACi release observed at all pH (Table 1), release in cells could be obtained at any stage of the penetration steps from direct water attack. SAHA and its derivatives **4b–c** presented equivalent EC_{50} (0.42 ± 0.08 μM, 0.59 ± 0.18 μM, and 0.75 ± 0.07 μM, respectively) indicating an effective release of the inhibitor from **4b–c** as expected while compound **4a** presented higher EC_{50} (7.17 ± 3.19 μM: Table 2) in agreement with the observed stability at all pH (Table 1). Thus, for SAHA derivatives, a good correlation is observed between acidic sensitivity and BRET induction.

CI-994 and its derivatives **5** gave also dose-dependent BRET induction with a sigmoid profile. (Fig. 2, CI-994 top left, compound **5** bottom right). Again prodrugs **5** are supposed to be inactive until CI-994 is released as CI-994 is attached by the orthobenzamide group suggested as the zinc binding group for this group of HDACi. CI-994 and compounds **5a–c** presented EC_{50} (0.86 ± 0.08 μM, 6.20 ± 1.37 μM and 0.68 ± 0.12 μM, 8.88 ± 3.89 μM respectively) not clearly in agreement with pH response in Table 1. This result could be due to difference in permeability for compounds **5a** and **5c** compared to **5b** which could be equilibrated by the respective pH sensitivity once the compounds are in the cells. The BRET signal being obtained from the nucleus, the diffusion across the nucleus membrane should also be considered.

As opposed to SAHA which is acidic and can give anionic form in biological pH, the free amino group of CI-994 is moderately basic and may be partly protonated in acidic part of the cells. Thus, if compounds **5** effectively release CI-994 in acidic compartments, CI-994 can possibly be retained in these compartments. Such acidic protonation of basic therapeutic agents is known and endosomal trapping can be an issue.

3.4. Kinetics of restoration of HDAC inhibition measured by BRET assay

The kinetics of the HDACi release in living cells were also determined by BRET assays after different times of incubation with the compounds. Fig. 3A shows the results obtained with free or linked SAHA. For all SAHA derivatives, maximal BRET signal inductions were obtained after 24–36 h of treatments. However, intensities of the induced BRET signals were different. Indeed, SAHA induced the higher increase in BRET signal (93.9 ± 7.9 mBu) compared to **4c** (66.2 ± 10.3 mBu), **4b** (44.3 ± 8.2 mBu), and **4a** (15.8 ± 4.9 mBu).

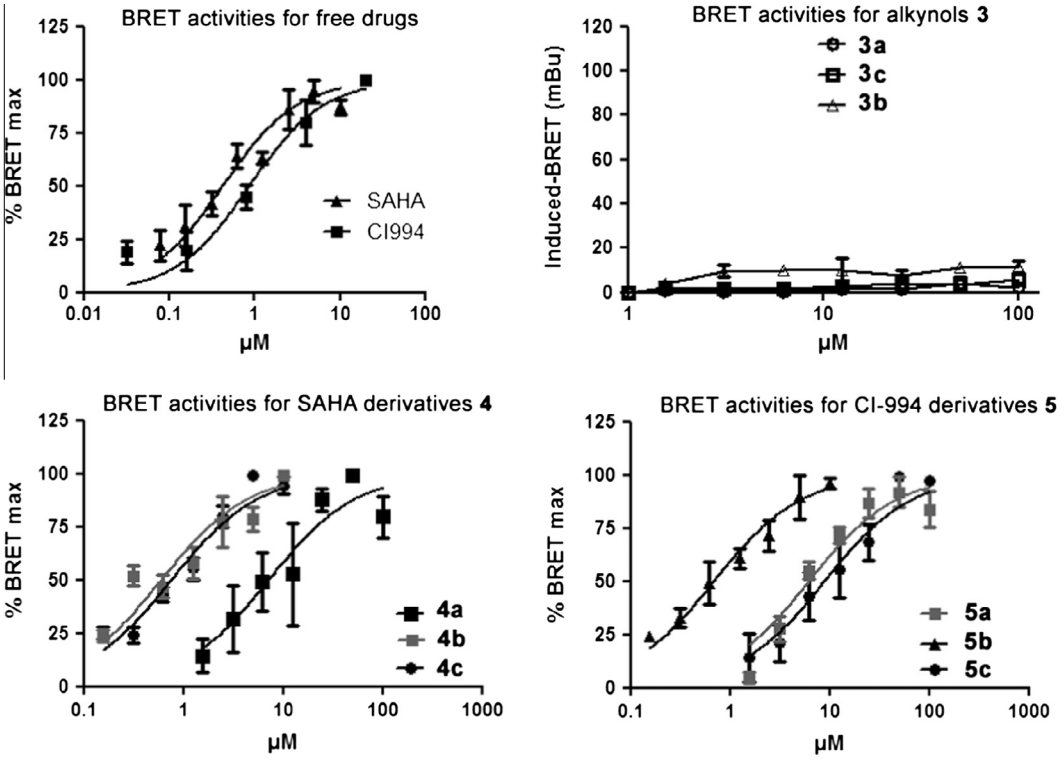

Fig. 2. Dose-dependent pharmacological characterization using BRET for free drugs, their derivatives 4 and 5, and the expected released alcohols 3. MeT-5A cells were transfected with phRluc-C1-BrD and pEYFP-C1 histone H3 and treated for 16 h with increasing doses of the different compounds. Then, BRET signals were measured as described in the materials and methods section. 100% corresponded to the maximal induced BRET signal measured. Results are the means ± SEM of three independent experiments.

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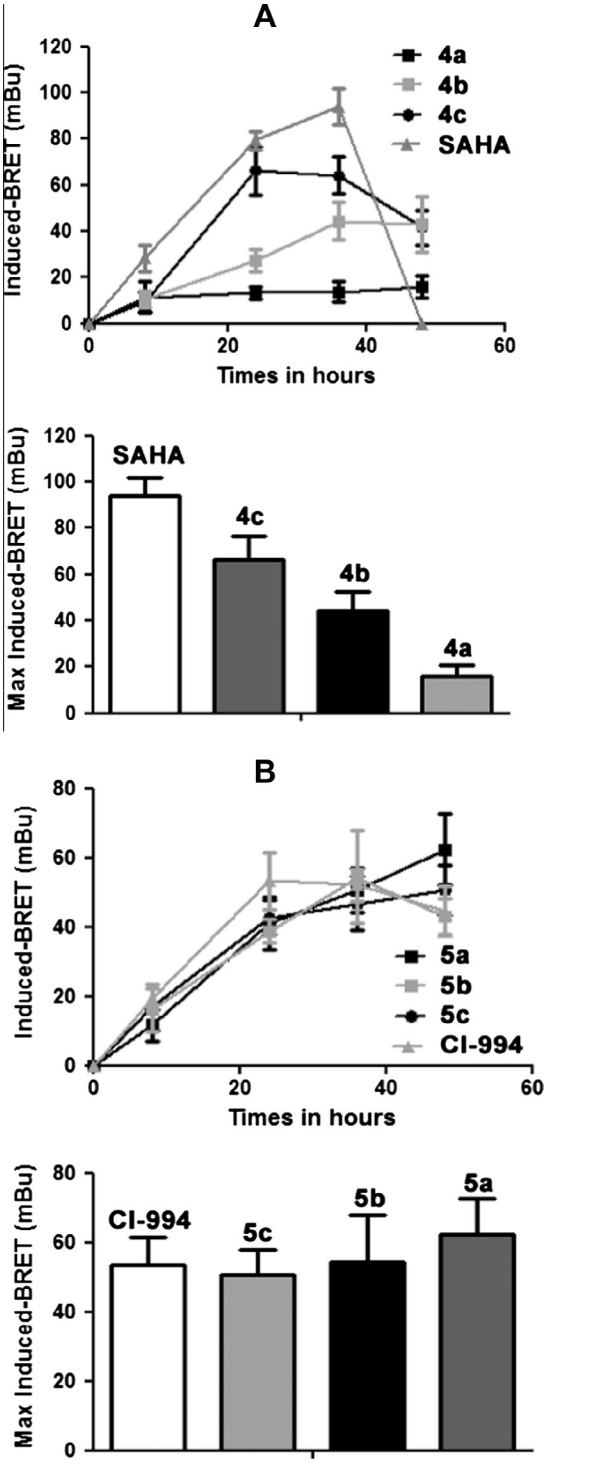

Fig. 3. Kinetic evaluation of the induction of histone H3 acetylation using BRET by: (A) SAHA and **4a–c**, (B) CI-994 and **5a–c**. (Top) Results are expressed as the induced BRET signal. (Bottom) Graphics represent the maximal induced BRET signal measured for each compounds. Results are the means ± SEM of three independent experiments. MeT-5A cells were transfected with phRluc-C1 BrD and pEYFP-C1 histone H3. Cells were treated during 8, 24 h, 36 h, or 48 h with 5 μM SAHA and **4a–c** (A) and with 10 μM CI-994 and **5a–c** (B). BRET signals were measured at the end of the treatment as described in the BRET measurements section.

Fig. 3B shows the results obtained with free or linked CI-994. Maximal BRET signal inductions were obtained for **5c**, **5b,** and CI-994 after 24–36 h of treatment. 48 h of treatment was needed with **5a** to obtain the maximal induction of BRET signal. No significant differences were observed for the maximal induction of BRET signal for all

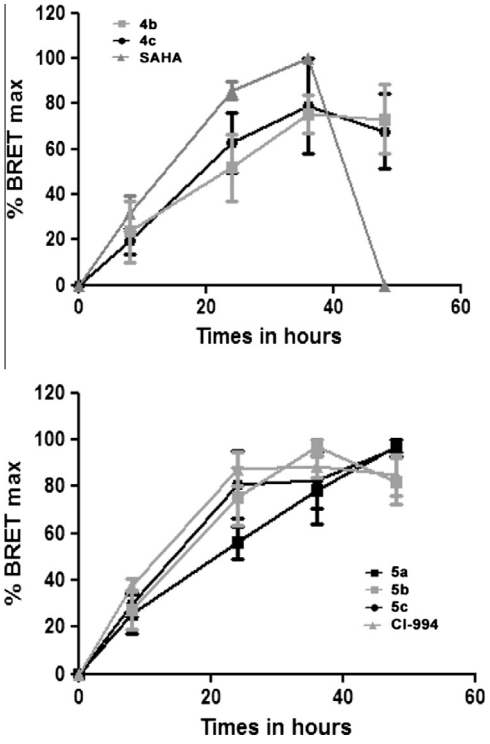

Fig. 4. Kinetic of induction of BRET signal by free drugs, their derivatives **4** and **5**. Data are presented as percent of maximal induced BRET. Left: SAHA and derivatives **4**. Right: CI-994 and derivatives **5**. The method was the same as for Fig. 2. Graphic represents the percent of maximal induced BRET signal measured for each compounds. Results are the means ± SEM of three independent experiments.

CI-994 derivatives (CI-994: 53.3 ± 8.2 mBu, **5a**: 62.3 ± 10.2 mBu, **5b**: 54.3 ± 13.3, **5c**: 50.6 ± 7.3 mBu).

As shown in Fig. 3A, the BRET induction for SAHA is faster and higher than the other derivatives **4**, and only compound **4c** seemed to have a maximum induction at shorter time. This can be the result of a partial early release of SAHA from **4c** out of the cells with subsequent diffusion inside cells, but in a smaller extent than SAHA alone, combined with a fast cell penetration of **4c** and subsequent SAHA release. For compound **4b**, a softer profile is found regarding the induction of the BRET signal, in agreement with a prolonged release over time in cells. Considering the penetration properties of the compound **4b**, this delayed BRET induction, reaching a plateau in our experiment, could also be explained by slower penetration in cells. The low BRET signal for compound **4a** could be due to its stability or its very slow cell penetration. Obviously, for SAHA, acidic susceptibility and cell penetration properties can be two ways to consider the prodrugs activities.

CI-994 and its derivatives demonstrated equivalent BRET responses, indicating that the release observed in acidic conditions for all derivatives (Table 1) is maintained in cells leading to fast release of CI-994 outside cells or in acidic compartments of the cells. Therefore, we hypothesized that an acidification-mediated release can be involved based on the time required for signal induction of the prodrugs when compared to their stability in simulated physiological pH conditions (>3 days for compounds **4a** and **5b**). Comparison between all the compounds in relative BRET induction mode is presented in Fig. 4, showing that the relative activity profiles are similar for all compounds. Only compound **5a** presented a relative slower release of the HDAC inhibitors in accordance with its hydrolysis rate determined in buffered solution.

A good correlation between BRET induction over time and pH response was thus observed for SAHA derivatives while for CI-994 prodrugs, the differences between the 3 versions were not significant.

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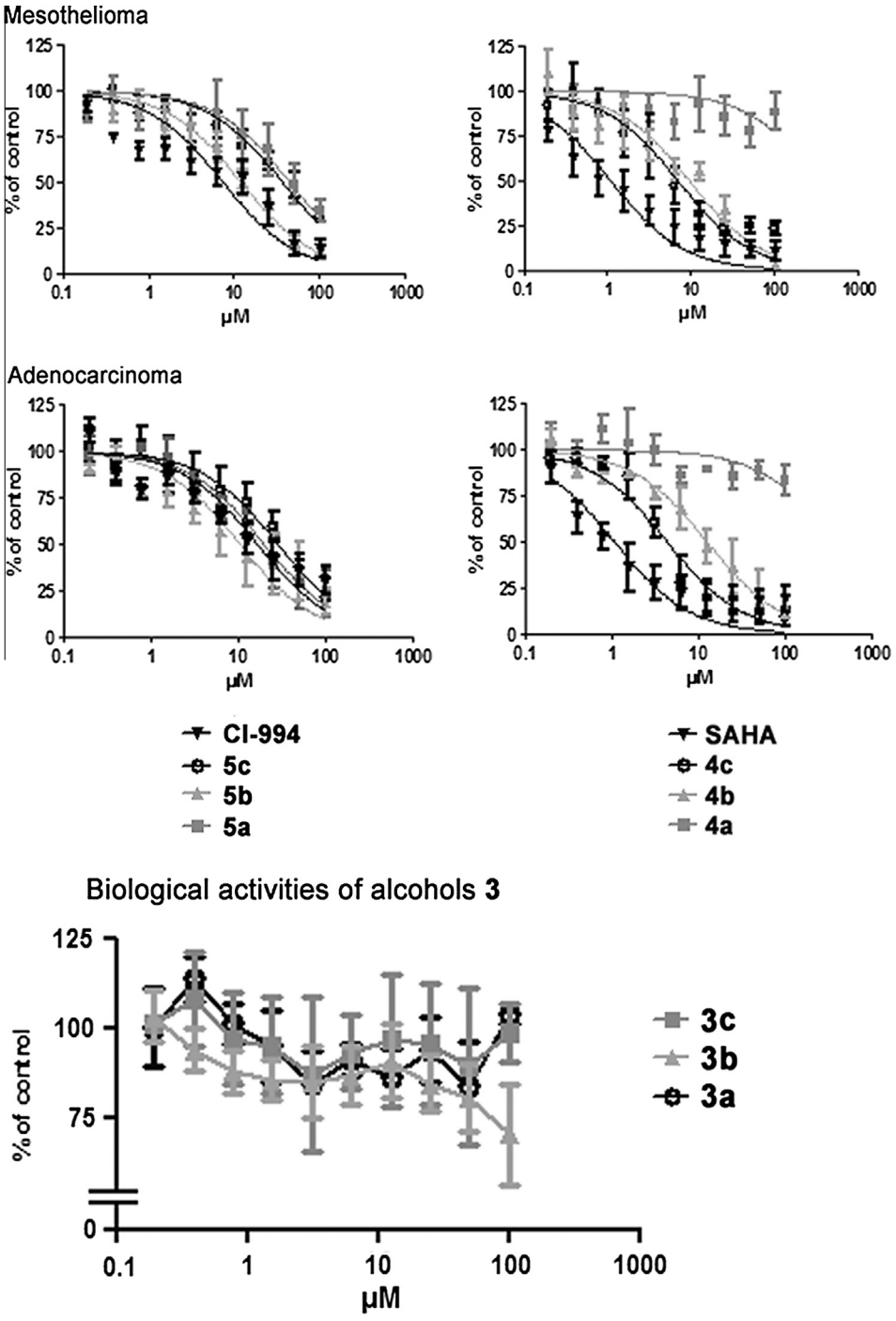

Fig. 5. Dose-dependent effect on MPM and lung ADCA cell viability for free drugs and derivatives 4, 5, and 3. MPM (Meso 13 and Meso 34) and lung ADCA (ADCA 72 and ADCA 153) cells were treated with increasing doses of CI-994, **5a–c**, SAHA, **4a–c**, or **3a–c** for 72 h. Cell viability was evaluated using Uptiblue cell counting reagent. Cell viability was expressed as the percent of control. Results are the means ± SEM of four independent experiments performed on two MPM or two lung ADCA cell lines.

3.5. Determination of cancer cell viability after treatments with HDAC inhibitors and their prodrugs

The correlation between histone H3 acetylation and toxic effect was determined for all compounds by viability tests on MPM and lung ADCA cells treated with increasing doses for 72 h (Fig. 5 and Table 3). All compounds, except **4a**, were toxic for MPM and lung ADCA cells depending on the concentration used. No major differences in half maximal inhibitory concentration (IC_{50}) were observed between MPM and lung ADCA cell lines for all compounds tested (Table 3).

No toxicity was obtained with compounds **3** at the IC_{50} concentration found for the free inhibitors (Fig. 5), confirming that the activities are only due to the release of HDACi from prodrugs.

Lower IC_{50} was obtained with SAHA on MPM and ADCA cell lines (1.47 ± 0.65 µM and 1.71 ± 0.94 µM, respectively). Intermediates IC_{50} were obtained with **4c** and **4b,** whereas **4a** did not affect cells viability. These results are in agreement with the three types

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Table 3
IC_{50} values for HDACi-induced inhibition of cell growth.

Compounds	IC_{50} (μM) MPM	IC_{50} (μM) ADCA
SAHA	1.47 ± 0.65	1.71 ± 0.94
4a	/	/
4b	9.39 ± 2.68	17.32 ± 6.93
4c	4.11 ± 0.59	5.22 ± 1.71
CI-994	14.49 ± 6.29	27.22 ± 10.35
5a	61.69 ± 29.72	29.05 ± 10.58
5b	12.46 ± 3.91	24.46 ± 13.66
5c	40.25 ± 12.85	29.4 ± 7.96

IC_{50} values were determined using GraphPad prism, Prism 5 for Windows, by curve fitting using a sigmoidal dose–response model. Results are the means ± SEM of four independent experiments performed on two MPM or two lung ADCA cell lines.

of prodrugs for SAHA, considering both pH response and cell penetration.

CI-994 and compounds **5a–c** presented similar IC_{50} on the cell lines tested in agreement with maximum BRET induction and fast release.

4. Conclusions

A synthesis of clickable pH responsive releasing system has been achieved, applied to two different functional groups found in HDAC inhibitors. The compounds were found stable when stored neat or when stored in DMSO solutions at −20 °C. Except for the series **c**, which is not stable whatever the pH conditions, the other series gave interesting prolonged release over time in buffered conditions. This was correlated with the in vitro slower restoration of HDAC inhibition as determined by BRET assays. Due to the type of releasing system and to the evident penetration of the prodrugs into cells (BRET is measured in cells), endocytosis could be involved, although co-localization of the molecules with acidic vesicles could not be determined with such molecules due to lack of any detectable properties. This internalization is also correlated with toxic activities toward cancer cell lines. Systems **4** and **5** are by themselves interesting intermediates as therapeutic agents and the released alcohols **3** appeared inactive in our tests at the usual IC_{50} concentrations for SAHA and CI-994. Indeed, because linked HDACi are not active, prodrugs should display no or weak hematological toxicities due to intravenous administration and release of the HDACi should occur mainly in tumor cells or in tumoral tissues where acidity is usually higher. Therefore, these compounds should decrease side effects associated with the use of HDACi in clinical trials [10] and increase their clinical benefits. These hypotheses would be verified on mouse models of tumor to compare efficacy of free or linked HDACi. The prolonged effect appeared very predictive when applied to SAHA. Considering the releasing mode of these prodrugs, it is clear that the oral route will not be feasible regarding the stomach acidity, except considering molecules where stomach release is wanted and provided that the released alcohols **3** have no effect in the stomach. In their current form, such prodrugs could be used for parenteral administration and the remaining alkyne group used to obtain specific applications. In particular, in vivo imaging could be of high interest to determine the biodistribution of the prodrugs. We also planned to use these alkyne prodrugs associated with azido-vectors (nanoparticles) able to reach the tumor site by the enhanced permeability and retention effect and also able to enter cells by endocytosis, where the acidity of the endosomes/lysosomes will be exploited to release the active compounds. The in vivo evaluation of such functional nanoparticles (biodistribution, pharmacokinetic, toxicity, and anti-tumoral activity) will be the next challenge. Alternatively, these molecules **4** and **5** could be used for in vitro fundamental epigenetic researches when longer HDACi effects (or other epigenetic targets) are required.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2013.03.006.

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