ACS Medicinal Chemistry Letters

Featured Letter

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Novel Polyamine−Naphthalene Diimide Conjugates Targeting Histone Deacetylases and DNA for Cancer Phenotype Reprogramming

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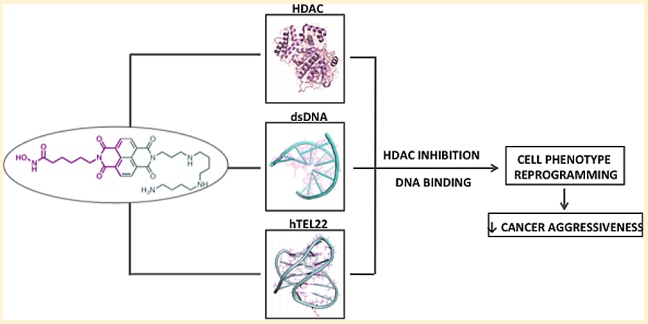
Supporting Information

ABSTRACT: A series of hybrid compounds was designed to target histone deacetylases and ds-/G-quadruplex DNAs by merging structural features deriving from Scriptaid and compound **1**. Compound **6** binds different DNA arrangements, inhibits HDACs both *in vitro* and in cells, and is able to induce a reduction of cell proliferation. Moreover, compound **6** displays cell phenotype-reprogramming properties since it prevents the epithelial to mesenchymal transition in cancer cells, inducing a less aggressive and migratory phenotype, which is one of the goals of present innovative strategies in cancer therapies.



KEYWORDS: *Multiple ligands, histone deacetylase, G-quadruplex, epithelial−mesenchymal transition*

The postchemotherapy occurrence of relapse and metastasis in many cancer types has encouraged research of novel tumor cell phenotype reprogramming strategies in order to improve patient response and survival. Since the expression and/or activity of epigenetic modulator enzymes are often deregulated in cancer, consistently with patient prognosis, chromatin modifier enzymes, influencing the chromatin transcriptional layout associated with a neoplastic phenotype, are gaining considerable interest as targets of these approaches.[1]

Several molecules have been used either alone or in combination with other anticancer agents to target the chromatin-mediated transcriptional control of gene expression, showing promising results in preclinical studies. Just a few of them, however, have gained actual clinical significance, certified by the Food and Drug Administration (FDA) with approval for the treatment of specific cancer subtypes. Among these epigenetic drugs, molecules targeting histone deacetylases (HDACs) are extensively studied as therapeutic agents in different diseases. As an example, Scriptaid, a naphthalimide-based HDAC inhibitor (HDACi), showed promising anticancer activities.[2]

HDAC inhibition has been reported to affect cancer cells mainly via a global relaxation of chromatin structure driving the unlocking of promoter regions typically controlling relevant epigenetically silenced tumor suppressor genes (TSGs). The following modulation of cancer cell behavior determines cell reprogramming toward a less aggressive phenotype, consistent with a recovered cell response to standard chemotherapeutic treatment, such as DNA-alkylating agents and topoisomerase inhibitors.[3] This observation highlights the concept that, as cancer cells exploit multiple and redundant biochemical pathways to ensure their survival, their treatment with multiple agents hitting distinct targets involved in the neoplastic development may represent a successful therapeutic approach. However, concomitant administration of different drugs with different pharmacokinetic and pharmacodynamic properties might not result in a true synergistic effect. The design of multiple ligands (MLs) appears as a promising alternative to

Received: July 18, 2017
Accepted: October 24, 2017
Published: October 24, 2017

DOI: 10.1021/acsmedchemlett.7b00289
ACS Med. Chem. Lett. 2017, 8, 1218−1223

combination therapy since it displays several advantages.[4] In particular, HDACis are extensively explored as components of MLs due to their above-mentioned synergy with chemotherapeutics. Furthermore, the binding site of HDACs allows the accommodation of a diverse array of chemical structures. In this respect, the development of MLs able to bind both HDACs and DNA seems to be a promising approach. Interesting results have been achieved with HDAC/topoisomerase inhibitors and with HDACi/Pt complexes, both of them being classes of compounds interacting with double-strand DNA (dsDNA). It is worth noting that, in addition to dsDNA, other secondary structures with relevant biological roles exist, such as the G-quadruplex DNA.[5] G-quadruplex appears to play a critical role in a range of biological processes since quadruplex-forming sequences are located not only in telomeric regions of the human genome[6] but also at the promoter region of several oncogenes. None of the HDAC-based MLs developed so far displays the ability to interact with both ds- and G-quadruplex DNA. Recently, our research group developed a series of naphthalene diimide (NDI)-based G-quadruplex binders.[7,8] Among these agents, the asymmetric NDI **1** emerged as the most promising antiproliferative agent. Compound **1** is characterized by two different chains mounted on the NDI scaffold: in detail, an *o*-methoxybenzylamino-propyl moiety on one side, and a spermine tail on the other side (Figure 1). NDI

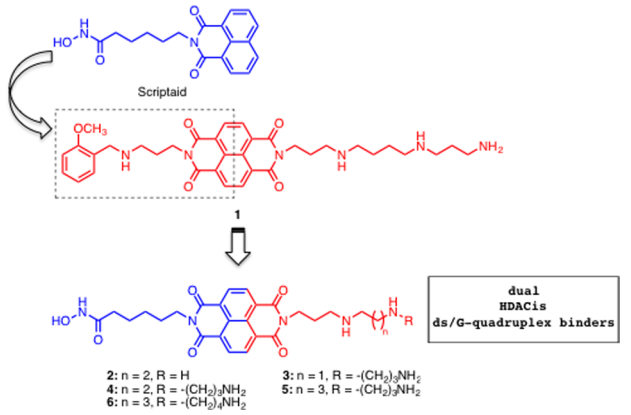


Figure 1. Drug design strategy leading to compounds **2**–**6**.

derivative **1** is endowed with a good affinity toward both human G-quadruplex and dsDNA, together with good antiproliferative and proapoptotic activities in different cancer cell lines.[9] Motivated by these considerations and by the structural similarity between compound **1** and the HDACi Scriptaid, hybrid compounds **2**–**6** were rationally designed by merging the structural features of the parent compounds. Indeed, we envisage that this combination may create compounds able to both bind DNAs and inhibit HDACs. The new compounds **2**–**6** are characterized by the presence of (a) a classic hydroxamic acid, as Zn^{2+}-binding group in place of the *o*-methoxybenzyl group, (b) an NDI moiety, allowing DNA interaction by stacking its large heteroaromatic surface, and (c) a polyamine chain varying in the methylene groups separating the amine functions. The cationic charges of the polyamine chain is intended to improve the molecular interaction with the negatively charged nucleotides and amino acids, as it has been already shown for other polyamine-based anticancer agents.[10,11] Herein, we report the evaluation of the potential multitarget profiles of compounds **2**–**6** by assessing their ability to bind both ds- and G-quadruplex DNA, to inhibit HDAC activity and to have a consistent functional effect over cancer cell phenotype.

Detailed descriptions of the synthetic sequences are reported in the SI (Schemes 1SI–4SI).

Fluorescence melting experiments on a human telomeric quadruplex sequence (hTel22) and on a duplex DNA sequence (dsDNA) were used to evaluate the target compound's (**2**–**6**) ability to bind both ds- and G-quadruplex DNA secondary structures. Thermal stabilization induced by tested compounds at 0.75 and 2.5 μM concentrations are reported in Table 1.

Table 1. ΔT_m Values (°C) for a Human Telomeric Quadruplex Sequence (hTel22) and Duplex DNA (dsDNA), and IC_{50} Values for *in Vitro* HDAC Inhibition in HeLa Nuclear Extract

	ΔT_m (° C)[b]				
	dsDNA		hTel22		
compd[a]	0.75 μM	2.5 μM	0.75 μM	2.5 μM	HDAC IC_{50} (μM)[c]
1	0.6	6.7	1.5	21.4	n.d.
2	1.0	3.3	2.4	6.3	0.41 ± 0.03
3	2.0	3.4	4.4	7.8	0.55 ± 0.02
4	5.0	8.5	9.9	14.5	0.37 ± 0.04
5	3.5	8.1	4.5	10.9	0.52 ± 0.03
6	7.5	14.0	13.3	20.4	0.56 ± 0.04
BRACO-19	0.5	5.3	11.0	28.3	n.d.
Scriptaid	0.1	0.1	0.1	0.1	0.56 ± 0.03

[a]Compounds **1**–**6** as hydrochloride salts. [b]Errors were 0.4 °C. [c]IC_{50} values represent the concentration causing 50% of HDAC activity

With the exception of compounds **2** and **3**, all new compounds bind dsDNA more efficiently than the reference compound **1**. In particular, among the derivatives containing the longer polyamine chains, an interesting trend can be defined: **3** < **4** ≈ **5** ≪ **6**. This suggests the importance of the length of the spacers separating the amine groups. Indeed, by increasing the number of methylene groups (see **3** vs **4** and **5** vs **6**), the interaction is implemented with a leveling off occurring, moving from compound **4** to compound **5**. Likely, this reflects the flexibility required to properly localize the amine groups in order to optimize the ionic contacts with the phosphate backbone. When compared to compound **1**, compound **6** is more efficient in stabilizing dsDNA, whereas the substitution of the *o*-methoxybenzyl-propyl group impacts negatively on the telomeric G-quadruplex recognition. Indeed, with the exception of compound **6**, all the new derivatives turned out to be less efficient than compound **1** as G-quadruplex binders. Compound **2**, characterized by two nitrogen atoms in the side chain, is less active than compounds **3**–**6** characterized by three nitrogen atoms. No clear indications of the role of the distance between the inner nitrogen atoms could be obtained for this series of compounds. Indeed, similar to dsDNA, compound **4** (characterized by a three-methylene spacer) is the most active on G-quadruplex among compounds **3**, **4**, and **5** as reducing or increasing the number of methylenes leads to a drop in the ΔT_m. Surprisingly, concerning G-quadruplex recognition activity, compound **6** shows a ΔT_m value comparable with that of compound **1** (Table 1, ΔT_m at 2.5 μM: compound **1**, 21.4 °C, compound **6**, 20.4 °C). In summary, all new derivatives are generally less specific for G-quadruplex in comparison to **1** or to the G-quadruplex binder Braco-19, used

DOI: 10.1021/acsmedchemlett.7b00289
ACS Med. Chem. Lett. 2017, 8, 1218–1223

as a reference. Consistently, all our new derivatives retain the ability to impair the activity of DNA processing enzymes (i.e., Taq-polymerase, Figure 1SI). The same analysis has also been performed using Scriptaid to exclude any direct interaction of this HDACi with DNA.

The *in vitro* ability of compounds **2**–**6** to inhibit HDACs has been preliminarily screened in HeLa nuclear extract (0.2, 0.5, 1, 5, and 25 μM concentrations) (Figure 2SI) and further explored in different cancer cell lines. Compounds **2**–**6** show IC_{50} values in the submicromolar range of concentrations, similar to the reference compound Scriptaid (IC_{50} = 0.56 ± 0.03 μM, Table 1) when HeLa purified nuclear HDAC enzymes are tested. To verify HDAC isoform selectivity, the inhibitory capacity of compound **6** has been evaluated with purified human HDAC1, 2, 4, and 6, representative of three HDAC classes (Table 2). Compound **6** displayed nanomolar activity against HDAC6 (IC_{50} = 0.05 ± 0.01 μM), while its activity against HDAC1, 2, and 4 was significantly lower.

Table 2. IC_{50} Values (μM) of Compound 6 against the HDAC1, 2, 4, and 6 Isoforms

	IC_{50} vs HDAC (μM)[b]			
compd[a]	HDAC1	HDAC2	HDAC4	HDAC6
6	4.12 ± 0.58	10.80 ± 0.10	117.00 ± 7.00	0.05 ± 0.01

[a]Compound as hydrochloride salt. [b]IC_{50} values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements.

However, testing HDAC inhibition in intact viable cells implies test compound internalization; thus, their effective concentration is expected to be affected (reduced) throughout their transport within the cell. The dose that completely inhibits HDACs in HeLa nuclear extract (5 μM) was used to evaluate the *in vitro* HDAC inhibitory activity of test compounds **2**–**6** in intact cells upon a 24 h treatment (Figure 2A). Cancer cell lines representative of different tissue types, i.e., glia (U87), breast (MCF7), colon (HCT116), and lung (A549) were used. Compounds **4** and **6** were able to inhibit at least about 40% of HDAC activity in U87, HCT116, and A549 cell lines with comparable effects, despite a different IC_{50} (compound **4**, 0.37 ± 0.04 μM, compound **6**, 0.56 ± 0.04 μM) scored in HeLa nuclear extract.

Compounds **4** and **6** resulted as significantly more active than Scriptaid in inhibiting HDAC activity in U87 and HCT116 cell lines ($p < 0.05$) while showing comparable effects to Scriptaid in the A549 cell line ($p < 0.05$). It is worth to note that compounds **2**–**6** and Scriptaid only slightly affect HDAC activity in MCF7 cells (Figure 2A). To further investigate the HDAC inhibitory activity of compound **6**, the global levels of histone H3 and H4 lysine acetylation (acH3 and acH4) were detected by Western blotting (WB) in A549 cells treated for 24 h at 5 μM concentration. The effect of compound **6** on histone acetylation was compared to that of compound **1**, which is defective of the hydroxamic acid moiety. The overall levels of acH3 and acH4 induced by compound **6** were two times higher ($p < 0.05$) with respect to control condition and compound **1**, confirming the HDAC inhibition capacity of compound **6** (Figure 2B,C).

Cell growth inhibition was evaluated at 0.2, 1, and 5 μM compounds **4** and **6**, as scalar concentrations (Figure 3SI), in the responsive U87, HCT116, and A549 cell lines. The effects were compared with that of compound **1**. Despite the observed

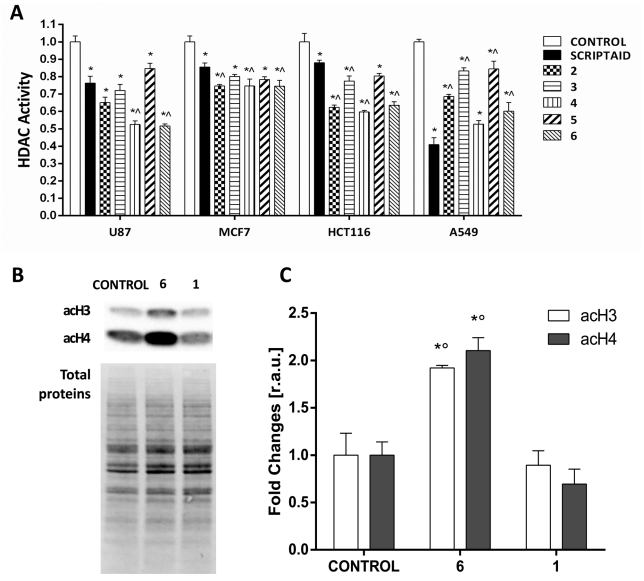


Figure 2. (A) HDAC inhibitory activity of compounds **2**–**6** compared to Scriptaid was tested with an *in vitro* cellular assay at a concentration of 5 μM for 24 h of treatment in U87, MCF7, HCT116, and A549 cell lines. The data are normalized with respect to the condition of the absence of inhibitor and reported as mean value ± SEM of three independent experiments (*$p < 0.05$ vs. control; ^$p < 0.05$ vs. Scriptaid;). (B) Global histone H3 and H4 acetylation (acH3, acH4) detected by WB in A549 cells treated with compounds **1** and **6** (5 μM) for 24 h. WB bands are shown out of one representative experiment, reporting the signal relative to acH3 and acH4 and the total amount of proteins per lane detected via a stain-free gel system (Bio-Rad). (C) Optical densitometry of acH3 and acH4 normalized with the total amount of proteins per lane. Reported data are the mean value ± SEM of three independent experiments versus control conditions (*$p < 0.05$ vs. control; °$p < 0.05$ vs 1).

comparable levels of HDAC inhibition in these distinct cancer cell lines (Figure 2A), compounds **4** and **6** induced different levels of reduction of cell proliferation, as witnessed by the GI_{50} values reported in Table 3 and determined by the inhibitory

Table 3. GI_{50} Values for Compounds 1, 4, and 6 in U87, HCT116 and A549 Cancer Cell Lines

	GI_{50} (μM)[b]		
compd[a]	U87	HCT116	A549
1	4.85 ± 0.05	4.39 ± 0.07	>5
4	>5	2.13 ± 0.07	>5
6	>5	3.08 ± 0.09	>5

[a]Compounds as hydrochloride salts. [b]GI_{50} values represent the concentrations causing 50% cellular growth inhibition. They were determined by the inhibitory dose–response curve, log(inhibitor) vs response with variable slope in GraphPad Prism 6.

dose–response curve (Figure 3SI). Although Scriptaid also shows slightly higher antiproliferative effect than compound **6** (data not shown), gene expression data (see below) suggests that survivor cells are not reprogrammed to a less aggressive phenotype, as it happens with compound **6** (Figure 4SI).

Modulating histone acetylation is likely to produce non-specific effects in a cell phenotype since it substantially relaxes chromatin architectures, allowing DNA consensus regulatory sequences to be accessed by their relevant transcriptional regulators. Epithelial–mesenchymal transition (EMT) has been

DOI: 10.1021/acsmedchemlett.7b00289
ACS Med. Chem. Lett. 2017, 8, 1218–1223

recognized as one among the adaptive changes known for being associated with cancer progression and metastasis formation.[12] EMT is a reversible process, which determines the phenotype transition of epithelial cells to a mesenchymal-like state responsible for the increased cellular motility, proliferation, and metastasis formation. Many studies showed that epigenetics is involved in the control of EMT,[13] and epigenetic drugs have been used for cell phenotype reprogramming.[14] The therapeutic potential of compound **6** has thus been evaluated studying its effect in reprogramming gene expression of cancer cells, monitoring the modulation of genes involved in the EMT, such as E-cadherin (*CDH1*), marker of the epithelial phenotype, and Vimentin (*VIM*), specific for the mesenchymal transition. The gene expression analysis of these two recognized EMT marker genes was performed upon a 48 h-long treatment with 5 μM **6** in HCT116 and A549 cell lines (Figure 3).

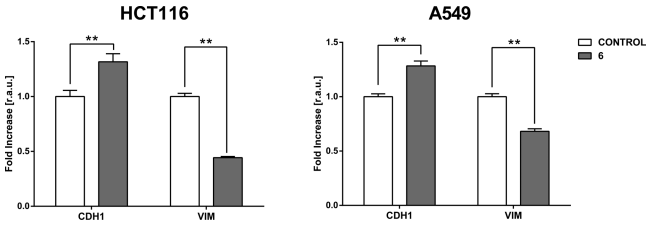


Figure 3. Gene expression analysis of *CDH1* and *VIM* genes in HCT116 and A549 cell lines treated with 5 μM compound **6** for 48 h. The data are reported as mean value ± SEM of four independent experiments (** $p < 0.01$).

Compound **6** upregulates *CDH1* and downregulates *VIM* expression in both cancer cell lines. *CDH1* level of expression was increased 1.32- and 1.28-fold, while *VIM* expression was consistently reduced 0.44- and 0.68-fold compared to the control condition in HCT116 and A549, respectively ($p < 0.01$). This effect credits compound **6** for being able to cell reprogram toward an epithelial phenotype, which is one of the goals of present innovative strategies in cancer therapies. It is worth to note that neither compound **1** nor Scriptaid induced effects comparable to compound **6** on *CDH1* and *VIM* expression in A549 cells (Figure 4SI). To further corroborate the role of compound **6** in the modulation of EMT transition, its effects were evaluated in A549 cells stimulated with transforming growth factor-β1 (TGF-β1) since the A549 cell line has been reported as an epithelial cellular model, switching to the mesenchymal phenotype when stimulated with TGF-β1.

Cell growth inhibition, gene expression analysis, and cell migration capacity were measured to this aim. As expected, A549 cells treated with TGF-β1 showed increased proliferation compared to unstimulated cells, as suggested by the increased amount of viable cells (Figure 4A). However, compound **6** significantly suppressed the TGF-β1-induced cell proliferation, with an 85% reduction of cellular growth that was comparable to what was observed with compound **6** alone (Figure 4A). Consistently, the level of expression of the EMT marker genes *CDH1* and *VIM*, which are respectively downregulated and upregulated by TGF-β1 stimulation compared to control condition, were reverted toward the control values when compound **6** was administered in combination with TGF-β1. In detail, *CDH1* expression resulted in 10 times downregulation by TGF-β1 compared to control condition. The administration of compound **6** in TGF-β1-treated cells induced a 3.66-fold *CDH1* upregulation (vs TGF-β1 alone). However, *VIM* showed

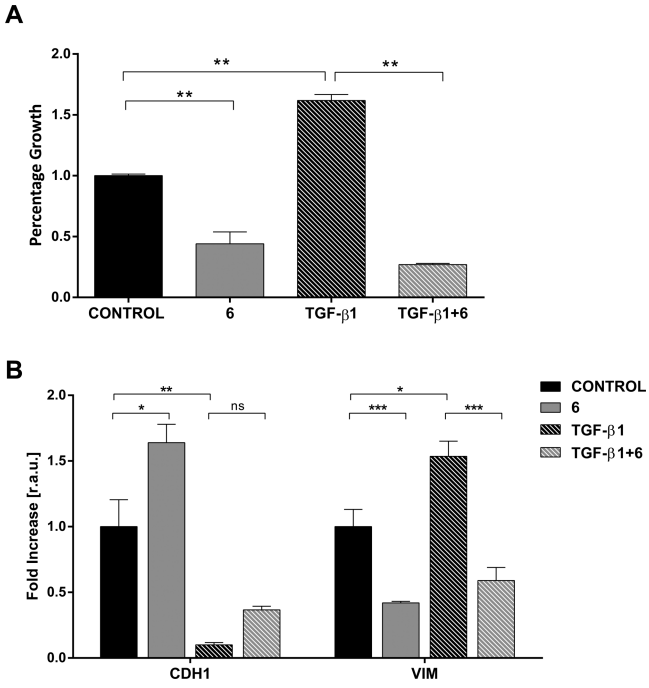


Figure 4. (A) Cell growth inhibition evaluated by compound **6** (48 h-long 5 μM treatment) in adherent A549 cell lines stimulated with TGF-β1. The percentage of growth was evaluated according to the NCI screening procedures, as mean value ± SEM of three independent experiments performed in triplicate. (B) Gene expression analysis of compound **6** effects on *CDH1* and *VIM* genes in A549 cell line stimulated with TGF-β1. Data are reported as mean value ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

the opposite behavior: TGF-β1 induced a 1.53-fold increase compared to control condition, and compound **6** administered in combination with TGF-β1 prevented this upregulation with a significant 2.61-fold reduction of the level induced by TGF-β1 alone (Figure 4B). Finally, in order to study how compound **6** modulated cell migration, a scratch wound healing assay was carried out in A549 cells stimulated with TGF-β1. As expected, TGF-β1 induced a significant increase of cell migration, determining 65% repopulation of the wound area at 24 h postscratch. However, unstimulated cells and cells treated with compound **6** alone maintained more that 80% of the wound area at 24 h. Interestingly, when compound **6** was administered in combination with TGF-β1, cells migration and proliferation were significantly (1.38-fold) reduced compared to TGF-β1 alone, confirming that compound **6** displays a promising ability of selection and/or reprogramming of cancer cells to a less aggressive phenotype (Figure 5).

A new drug design strategy is emerging based on the assumption that complex diseases, such as cancer, may be better faced by multiple ligands (MLs). Due to their relevant roles in cancer development, progression, and response to therapy, HDACs have been identified as interesting candidates to be targets of such MLs. In this preliminary report, we have developed hybrid compounds that merge HDAC inhibition properties with DNA recognition competence via hydroxamic acid and NDI moieties, respectively. Compound **6** emerges among these new NDI derivatives as the most promising hybrid, able to both bind DNA and inhibit HDACs. The multitarget effects of compound **6**, compared with the individual effects of its parental compounds **1** and Scriptaid,

DOI: 10.1021/acsmedchemlett.7b00289
ACS Med. Chem. Lett. 2017, 8, 1218−1223

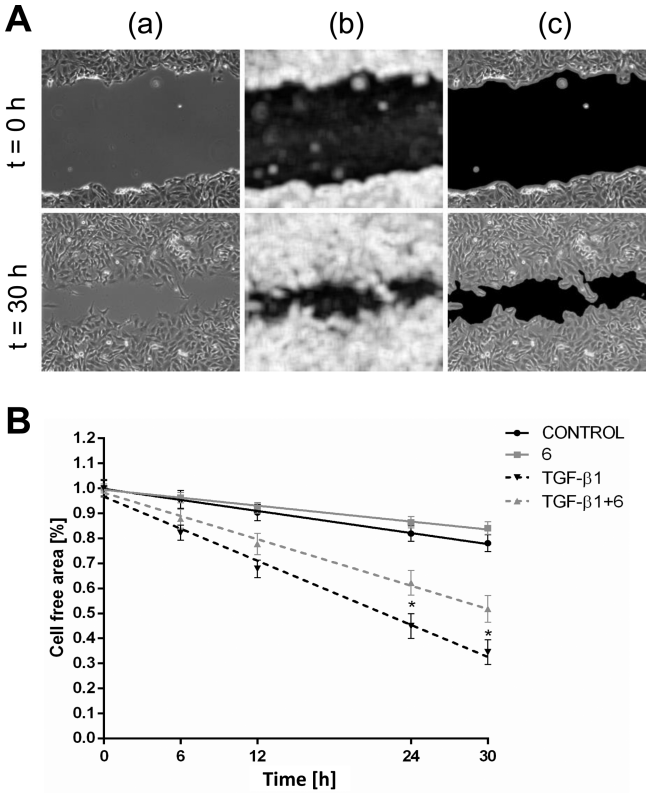


Figure 5. Scratch wound healing assay of the effect of compound **6** in TGF-β1 induced A549 cells. (A) Example of image processing comparing two different time points (t = 0 and t = 30 h): (a) raw image; (b) local entropy image; (c) final segmented image (see Materials and Methods). (B) Cell-free surface area was calculated over time and fitted with a linear regression. Data are reported as mean value ± SEM of three independent experiments. *p < 0.05.

are emphasized by its ability to induce a decrease of cell viability. Since compound **1** and Scriptaid affect cell proliferation, the effect induced by compound **6** is likely to be related to its double action of DNA binder and HDACi. Compound **6** displays also cell phenotype reprogramming properties not exhibited by neither compound **1** nor Scriptaid. This is evident by the reduction of cell migration consistent with the downregulation of the mesenchymal marker *VIM* and the upregulation of the epithelial marker *CDH1*. This effect credits compound **6** for being able to induce cell reprogramming toward a less aggressive epithelial phenotype, which is one of the goals of present innovative strategies in cancer therapy. The observed effects of compound **6** are clearly related to the combined interaction with HDAC and DNAs that can produce synergistic effects. Indeed, HDAC inhibition, by inducing hyperacetylation of histones, relaxes chromatin and exposes both ds- and telomeric G-quadruplex-DNAs to damaging molecules. Worth of mention, in addition to telomeres, G-quadruplex may form in additional genomic sites, i.e., at promotors of oncogenes, and again, HDAC inhibition may facilitate its stabilization by G-quadruplex binders and alter the gene expression profile. In this respect, further investigations are currently ongoing in our laboratories and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00289.

Synthetic procedures and characterization of compounds, and biological procedures (PDF)

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Author Contributions

▽These authors 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.

Funding

This work was supported by the University of Bologna (RFO) and the Italian Ministry for Education, Universities and Research (MIUR).

Notes

The authors declare no competing financial interest.

Biography

Andrea Milelli received his Master's degree in Chemistry and Pharmaceutical Technologies from the University of Bologna followed by a Ph.D. in Pharmaceutical Sciences in 2009. He spent a research period at Aarhus University, Denmark, working under the supervision of Prof. Karl Anker Jørgensen, and in 2016, he was Visiting Scientist at Konstanz University, Germany. Currently, he is Assistant Professor at the University of Bologna. His current research focuses on the development of small molecules with potential applications in cancer and neurodegenerative diseases.

■ ACKNOWLEDGMENTS

Authors thank Laura Fantini and Valentina Bondi for technical support. A.P., M.C., and E.G. are members of the COST action CM1406 (EpiChemBio).

■ ABBREVIATIONS

CDH1, E-cadherin; dsDNA, double-strand DNA; EMT, epithelial−mesenchymal transition; FDA, Food and Drug Administration; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; ML, multiple ligands; NDI, naphthalene diimide; TGF-β1, transforming growth factor beta 1; TSGs, tumor suppressor genes; VIM, Vimentin

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DOI: 10.1021/acsmedchemlett.7b00289
ACS Med. Chem. Lett. 2017, 8, 1218−1223