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Histone deacetylases as an epigenetic pillar for the development of hybrid inhibitors in cancer

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The polypharmacology strategy of multi-targeting drugs acting on different biological pathways is capturing the researchers' attention, particularly in cancer. The simultaneous inhibition of two or more targets by drug combination or by a single 'hybrid molecule' can provide improved therapeutic efficacy when compared to the one-target inhibitors. In this regard, because of their multiple anticancer effects, histone deacetylase inhibitors have become a privileged tool for the development of hybrid drugs. The clinical trials of two multi-acting chimeras, HDAC/EGFR/HER2 and HDAC/PI3K inhibitors, encouraged the design of novel hybrids, such as compounds **22a** (LSD1/HDAC) and **16a** (CDK4/JAK1/HDAC), which showed superior anticancer effects than single-targeting agents or their combination both in cellular and mouse models.

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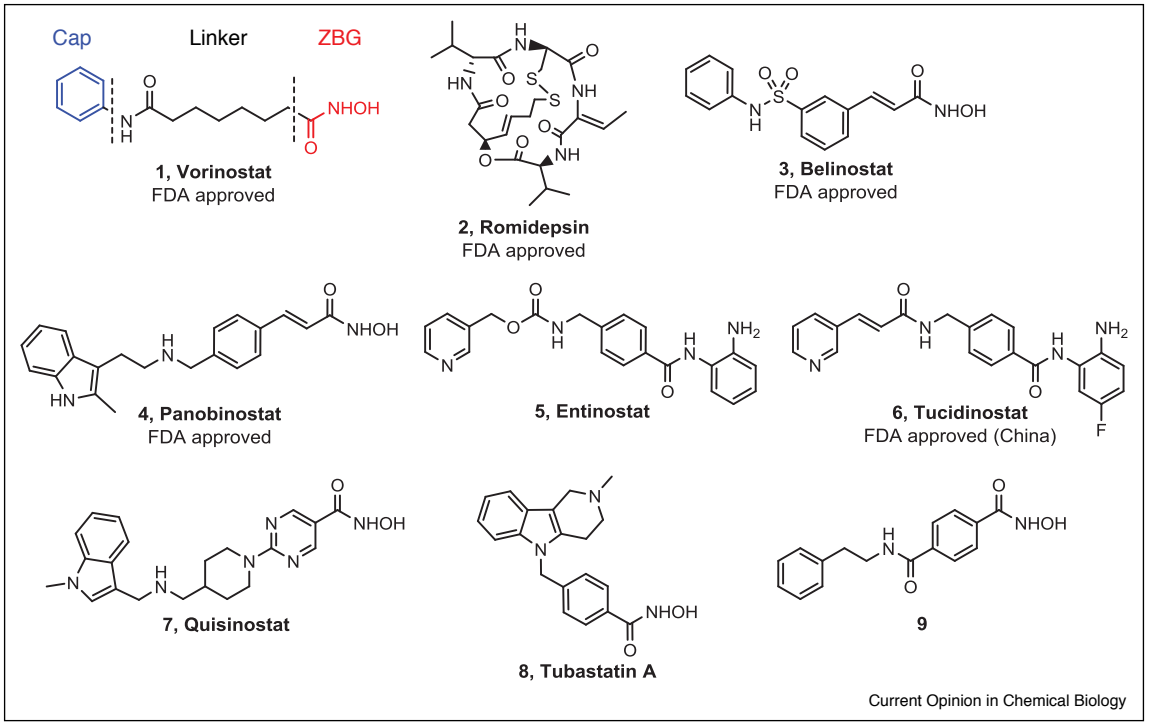
Introduction

It is now well established that cancer is a multi-factorial disorder caused by genetic and/or epigenetic alterations leading to the de-regulation and mis-regulation of numerous and different pathways via varied molecular mechanisms. In this scenario, three different approaches are being used in preclinical and/or clinical settings: (a) multi-targeting or promiscuous drugs showing a wide spectrum of biological activities with possible adverse reactions [1]; (b) the combination of two or more drugs acting on different targets; and (c) the more recent, smart hybridization of at least two different pharmacophore entities in a single drug; such so called 'chimeras' are able to simultaneously inhibit multiple cancer targets [2,3,4••,5]. Potential advantages of multi-targeting drugs are manifold. Hybrid drugs can benefit from a more predictable and less complex metabolism, more favorable pharmacokinetic and pharmacodynamic parameters, and improved bioavailability. Moreover, a hybrid compound guarantees the simultaneous presence of the two pharmacologically active entities in the same cell in contrast to the co-administration of two single synergistic compounds, which often reach the site of action with different efficiencies [2]. Additional benefits can be afforded by the improved patient compliance and reduced costs [6,7].

In the last two decades, histone deacetylase inhibitors (HDACi) emerged as very effective tools in cancer research and pharmacology. They activate multiple anti-tumor pathways, such as extrinsic (death receptors and ligands upregulation) or intrinsic apoptosis (anti-apoptotic factors downregulation, pro-apoptotic upregulation). Moreover, HDAC inhibitors target and/or modulate the expression of non-histone proteins including transcription factors and regulators, inflammation/immune response mediators, DNA repair enzymes, nuclear import regulators, chaperones, and structural proteins [8,9]. They further induce growth arrest (p21 upregulation, cyclins downregulation), mitotic and autophagic cell death, senescence, and anti-angiogenic effects (HIF-1α function and VEGF downregulation). So far, five HDACs inhibitors (HDACi) have been approved by the FDA for the treatment of hematological malignancies, though they remain poorly effective in solid cancers. The evidence that co-administration of antiHDAC molecules with other anticancer drugs can improve anticancer effects encouraged the design of HDACi-based hybrid molecules [2,3,4••,5]. Specifically, drug combinations have been sought to achieve enhanced efficacies, decreased dosage, reduced or delayed development of drug resistance, simultaneous enhancement of therapeutic actions, and reduction of side-effects [10,11]. HDACi thereby offer an excellent model system to investigate and validate the concept of multi-targeting hybrid drugs.

The general molecular features of the novel HDAC-based hybrids comprise (i) a 'cap' moiety acting against a cancer target [2,4••,5] (see **1** in Figure 1);

Figure 1



A summary of the HDAC inhibitors used for the design of multi-targeting hybrids including a schematic representation of the HDAC pharmacophore model.

(ii) a HDAC-inhibiting zinc-binding group (typically a hydroxamic acid or a 2-aminoanilide); and (iii) a linker joining the zinc-binding group to the cap (Figure 1). The main HDACi-based hybrids described in the recent literature include kinase inhibitors, cytotoxic agents, epigenetic modulators, hormone and vitamin D receptor modulators, natural products, and other anticancer drugs [4••]. In this opinion article, we will give an overview of the main work reported from 2016 to 2018 with a focus on the most interesting hybrid compounds active in cancer.

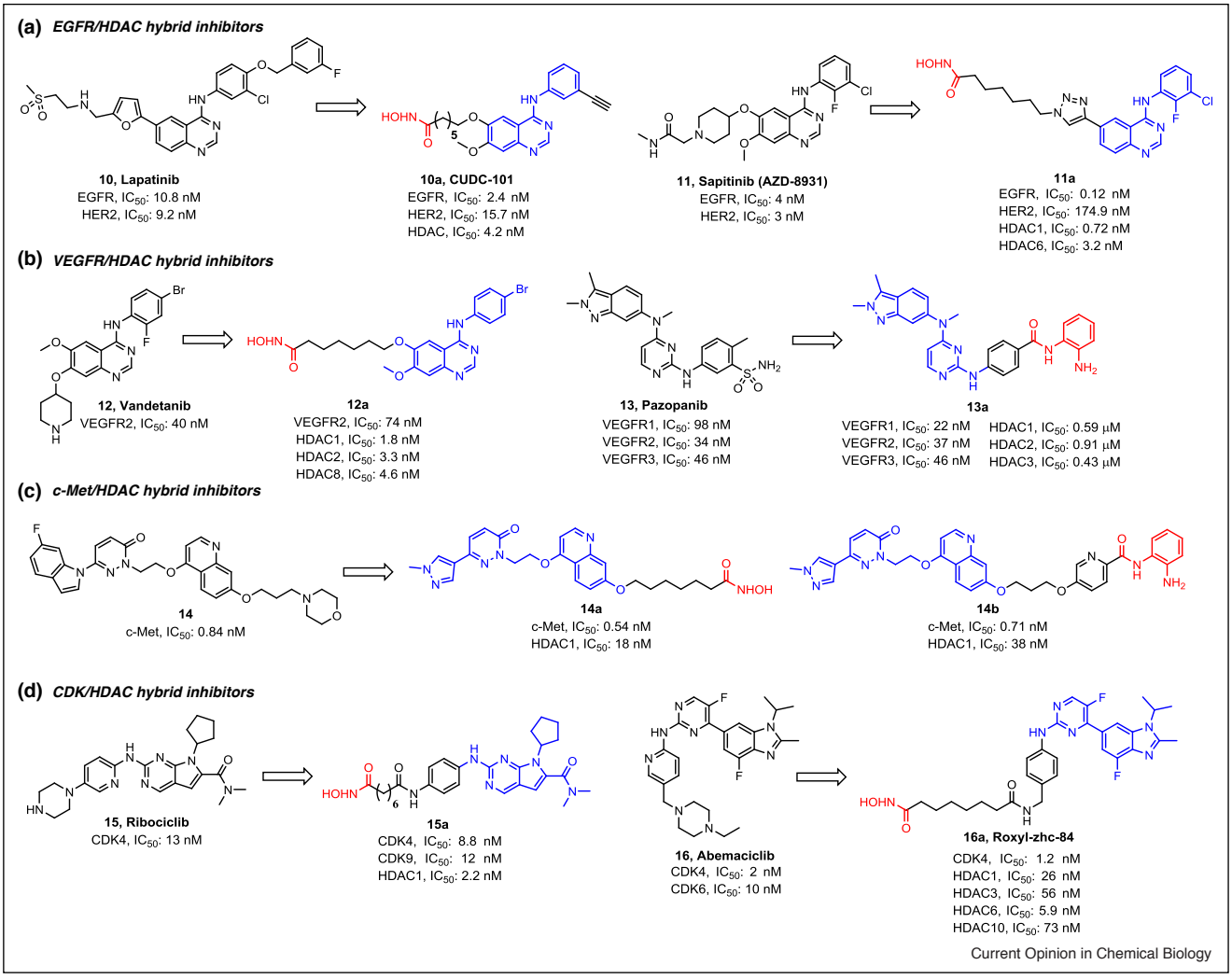
HDAC/kinases hybrid inhibitors

Kinases represent the most intensively studied classes of drug targets and their inhibitors are employed mainly for cancer treatment [5]. Many kinase inhibitors have been approved or are currently in clinical trials, although their efficacy is often impaired by the onset of drug resistance [12]. Since HDACi proved to synergize with kinase inhibitors by blocking proliferation, inducing apoptosis, and improving the sensitivity to anti-kinase drugs, researchers extensively attempted the development of HDAC/kinase dual-acting compounds [5]. A successful example is represented by the EGFR/HER2/HDAC hybrid inhibitor CUDC-101 (**10a**), the first-in-class and clinically studied chimera (NCT00728793, NCT01171924) (Figure 2). Compound **10a** displayed enhanced pro-apoptotic effects when compared to the combination of the single-targeting agents, erlotinib (EGFR/HER2 inhibitor) and vorinostat (**1**, a well-known HDACi, Figure 1), in erlotinib-resistant tumor cells [13]. Critically, **10a** was shown to achieve synergistic effects and overcome resistance by modulating multiple variables affecting receptor tyrosine kinase signaling, thereby suppressing survival and compensatory pathways [13].

EGFR/HDAC hybrid inhibitors

Prompted by the promising results obtained with **10a** and similar hybrids merging **1** to the 4-anilinoquinazoline core moiety of the dual EGFR/HER2 inhibitor sapitinib (**11**) [4••,5], Ding *et al.* described a novel series of hybrid antiEGFR/HDAC compounds [14] (Figure 2). Among this series, **11a** displayed nanomolar IC_{50} values and proved to be 3–80-fold more potent than gefitinib (EGFR IC_{50}: 9.6 nM) and **1** (HDAC1 IC_{50}:12 nM and HDAC6 IC_{50}:11 nM). In A549 and BT-474 cells, **11a** dose-dependently reduced phospho-EGFR levels and induced H3 hyperacetylation. Furthermore, **11a** strongly impaired proliferation in lung A549 cells (IC_{50}: 0.63 μM), being more efficient than **1** (IC_{50}: 2.57 μM) and lapatinib (**10**, IC_{50}: 1.74 μM), even though it was less potent against breast ductal BT-474 cells (IC_{50} values in μM: 3.88 for **11a**, 2.67 for **1**, and 0.10 for **10**).

Figure 2



Development of HDAC/kinases hybrid inhibitors **10a–16a**.

VEGFR/HDAC hybrid inhibitors

The 4-anilinoquinazoline core was further exploited for the development of VEGFR/HDAC dual inhibitors. Specifically, a novel hybrid series was obtained by merging the scaffolds of vandetanib (**12**) and **1** [15,16] (Figures 1and 2). Overall, the hybrid **12a** displayed the best inhibition profile towards both HDAC1 and VEGFR-2 when compared with single targeting agents **1** and **12** [16]. **12a** further exhibited sustained inhibition of MCF-7 breast cancer cells proliferation (IC_{50}: 0.85 μM), being 5-fold and 22-fold more efficient than **1** (IC_{50}: 4.2 μM) and **12** (IC_{50}: 18.5 μM), respectively. Moreover, since pazopanib (**13**) provided good results when used in combination with several HDACi [17], hybrid **13a**, bearing a 2-aminoanilide as the zinc-binding motif, was developed. **13a** showed a good inhibition profile against HDAC1-3 and VEGFR1-3, and displayed potent antiproliferative activities not only in hematological but also in solid tumor cell lines (e.g. HT-29 colon cancer, IC_{50} values: 1.07 (**13a**), 1.51 (**1**), 3.10 (**5**, entinostat in Figure 1), and >5 (**13**) μM). Notably, in HUVEC cells, **13a** (IC_{50}: 16 μM) showed improved or similar IC_{50} values compared to the combinations of **1** and **13** (IC_{50}: 6.9 μM) or **5** and **13** (IC_{50}: 27.3 μM) and was more cytotoxic than the single agents (IC_{50} values: 61.4 (**1**), 78.1 (**5**), and 33.5 (**13**) μM). Moreover, in HT-29 xenografted BALB/c nude mice oral administration of **13a** (50 mg/kg day) displayed a 40% tumor growth inhibition after 25 days. This level of tumor growth suppression was comparable to **13** (50 mg/kg day) or **1** (100 mg/kg day) [18•]. In HeLa cells **13a** (1 μM) increased H4 acetylation levels comparably or better than **5** at the same dose. In HUVECs, **13a** further inhibited VEGFR-2 phosphorylation and tube formation as effectively as **13**.

c-Met/HDAC hybrid inhibitors

Through a successful kinase/HDAC hybrid inhibitor design, Lu *et al.* described the first potent c-Met and HDAC dual hybrid binders [19] (Figure 2). The selective c-Met inhibitor **14** [20] served as a core scaffold for synthetizing the new hybrids. Among them, compound **14a** displayed nanomolar IC_{50} against both c-Met and HDAC1. **14b**, though less potent in biochemical assays, provided higher proliferation inhibition in the colon cancer cell line HCT-116 (IC_{50}: 1.3 μM) and in the lung cancer cell line EBC-1 (IC_{50}: 0.058 μM) cells when compared to **14a** ($IC_{50} > 10$ μM and 0.21 μM, respectively), and it was more or equally potent than the single targeting agents **1** (IC_{50}: 1.4 and 1.3 μM, versus HCT-116 and EBC-1 cells, respectively) or **14** ($IC_{50} > 10$ and 0.060 μM, respectively). Additionally, the authors studied the action mechanism of **14b**, which displayed inhibition of c-Met-mediated phosphorylation, as well as the induction of H3 hyperacetylation and p21 expression [20].

CDK/HDAC hybrid inhibitors

Thanks to the synergistic effects observed by combining HDAC with CDK inhibitors [21], Li *et al.* designed the new hybrid **15a**, obtained by merging the CDK4/9 inhibitor ribociclib (**15**) and **1** [22] (Figures 1 and 2). This hybrid inhibited HDAC1 and CDK4/9 with nanomolar IC_{50} values, being more potent than **15** against CDK4. When tested against a large panel of cancer cell lines, **15a** displayed a single-digit micromolar antiproliferative activity (ranging from 1.11 to 3.78 μM) and was thus slightly more effective than **1**. Furthermore, under the same assay conditions, **15** was not as potent as **15a**. In-depth cell studies confirmed that **15a** inhibited CDK4/9 and HDAC1, triggering apoptosis. Moreover, in 4T1 xenografted BALB/c female mice, intraperitoneal (18 days) or oral (24 days) administration of **15a** (130 mg/kg day) inhibited tumor growth by about 80% which was a similar or better outcome than observed in mice treated with **1** (~77%) or **15** (40–70%) at the same dosage.

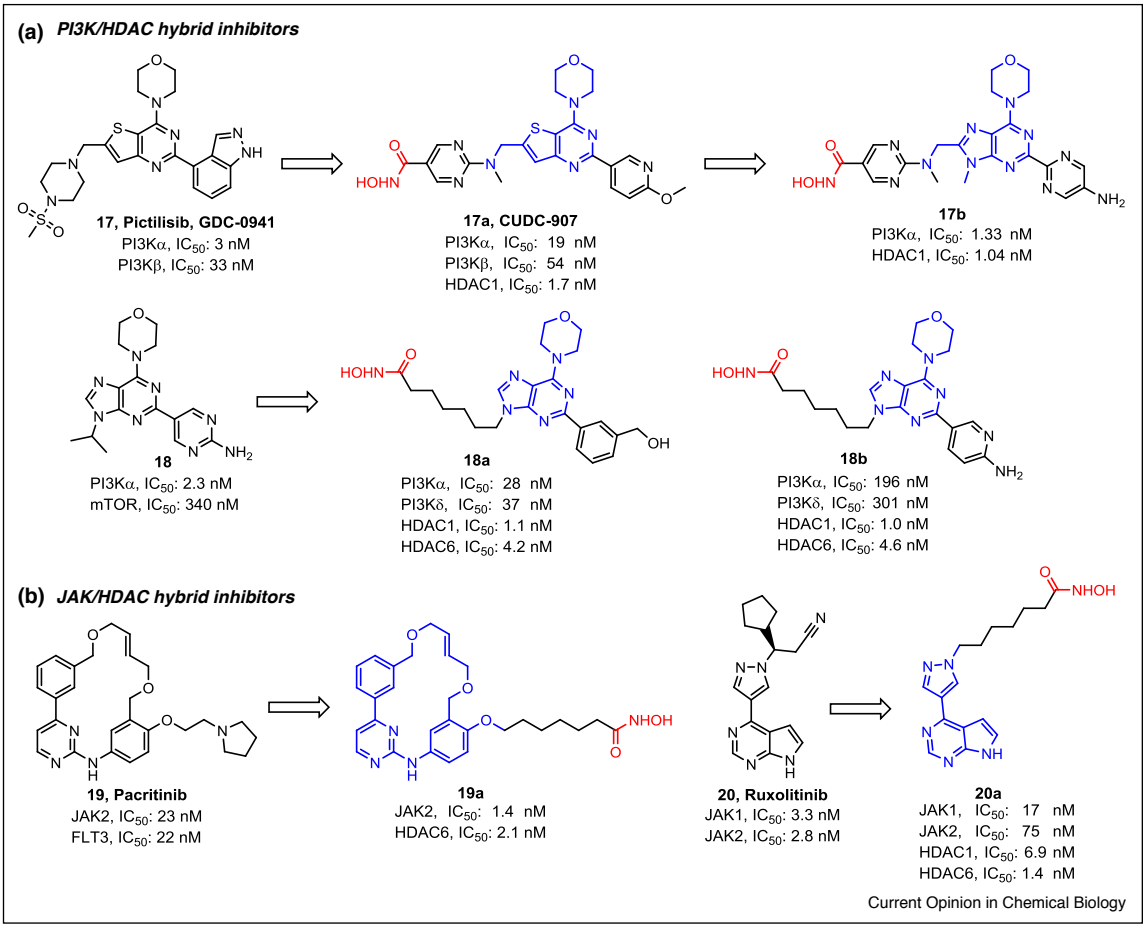
In an independent work, **1** was hybridized with abemaciclib (**16**), a CDK4/6 inhibitor, to afford a novel hybrid compound, Roxyl-zhc-84 (**16a**) that was able to simultaneously inhibit HDAC1-3, 6, 10, and CDK4 at nanomolar levels (Figure 2) [23]. **16a** was tested on a large panel of kinases, displaying nanomolar IC_{50} values against JAK1 and, similarly to **16** [24], significant inhibition of other targets at 1 μM (e.g. IKKα 80%, MSK1 84%). **16a** gave promising results when tested against various aggressive breast and non-small cell lung cancer cell lines (**16a** IC_{50} range 18.3–44 nM, **1** $IC_{50} > 1$ μM, **16** IC_{50} range 0.7–>2.0 μM). In MDA-MB-231 and MDA-MB-468 cells, **16a** was significantly (>2-fold) more effective than the combination of **1** and the JAK1 inhibitor filgotinib at inducing G1-phase arrest, caspase cleavage and p21 upregulation, suppressing JAK1, STAT3 and Rb phosphorylation, and downregulating BCL2. In three mouse cancer models (4T1-Fluc allografted or MDA-MB-231 xenografted or MDA-MB-468 xenografted BALB/c mice), oral administration (30–40 days) of **16a** (15/30/60 mg/kg day) led to significant tumor regression when compared to **1** (60 mg/kg day), **16** (60 mg/kg day), or to the combination of **1** and filgotinib (60 + 60 mg/kg day, tested in MDA-MB-231 xenografted mice), prolonging lifespan with negligible toxicity. Importantly, **16a** markedly improved the poor HDACi efficacy in solid tumors by overcoming JAK1-STAT3-BCL2-mediated drug resistance, and was more efficient than the combination of the single-target agents [25••].

PI3Ks/HDAC hybrid inhibitors

Currently, several PI3K inhibitors are in clinical trials as monotherapy or in combination with other drugs for cancer treatment, including pictilisib [26,27] (Figure 3). However, the clinical application of PI3K inhibitors is impaired by the concurrent activation of different pathways involved in cancer cell survival and/or growth. The evidence that the combination of HDACi with PI3K inhibitors provided synergistic anticancer effects [28,29] prompted Qian *et al.* to design the first in class PI3K/HDAC dual compounds, CUDC-907 (**17a**) [30], which is currently in a phase 2 clinical trial (NCT02674750) (see Refs. [4••,5]). Encouraged by the results shown by **17a**, Chen *et al.* synthesized a novel series of PI3K/HDAC hybrid inhibitors [31] (Figure 3). Among them, **17b** displayed a dual balanced single digit nanomolar PI3Kα and HDAC1 inhibition being 10-fold more potent than **17a** against PI3Kα. In MV4-11, A2780s, and HCT116 cells, **17b** (IC_{50} values 8, 117, and 214 nM, respectively) inhibited proliferation more potently than **1** (IC_{50} values 230, 870, and 1450 nM, respectively) although it was less effective than **17a** (IC_{50} values 0.4, 6.1, and 7.3 nM, respectively). **17b** induced H3 and H4 hyperacetylation and dose-dependently reduced the levels of mTOR, PI3K, and Akt phosphorylation. Hybrid **17b** (intravenous 10 mg/kg every two days) also proved good efficacy when tested in MV4-11 xenografted NOD/SCID mice with 45% tumor-growth inhibition after 11 days, whereas **1** (intraperitoneal 50 mg/kg every two days) displayed modest or no effects [31]. When evaluating these data, it must be noted that a **17b**-related compound displaying no PI3Kα inhibition, was more potent than **17b** both in cellular (MV4-11, A2780s, HCT116 IC_{50} 0.1, 7, 0.7 nM, respectively) and mice experiments (10 mg/kg, tumor-growth inhibition 65%) [31].

More recently, the same group developed another potent series of dual PI3K/HDAC inhibitors starting from the purine-based PI3K inhibitor **18** [32] (Figure 3). Among them, compound **18a** showed improved IC_{50} values against HDAC1/6 and PI3Kβ than the single-target compounds, as well as stronger or equal antiproliferative

Figure 3



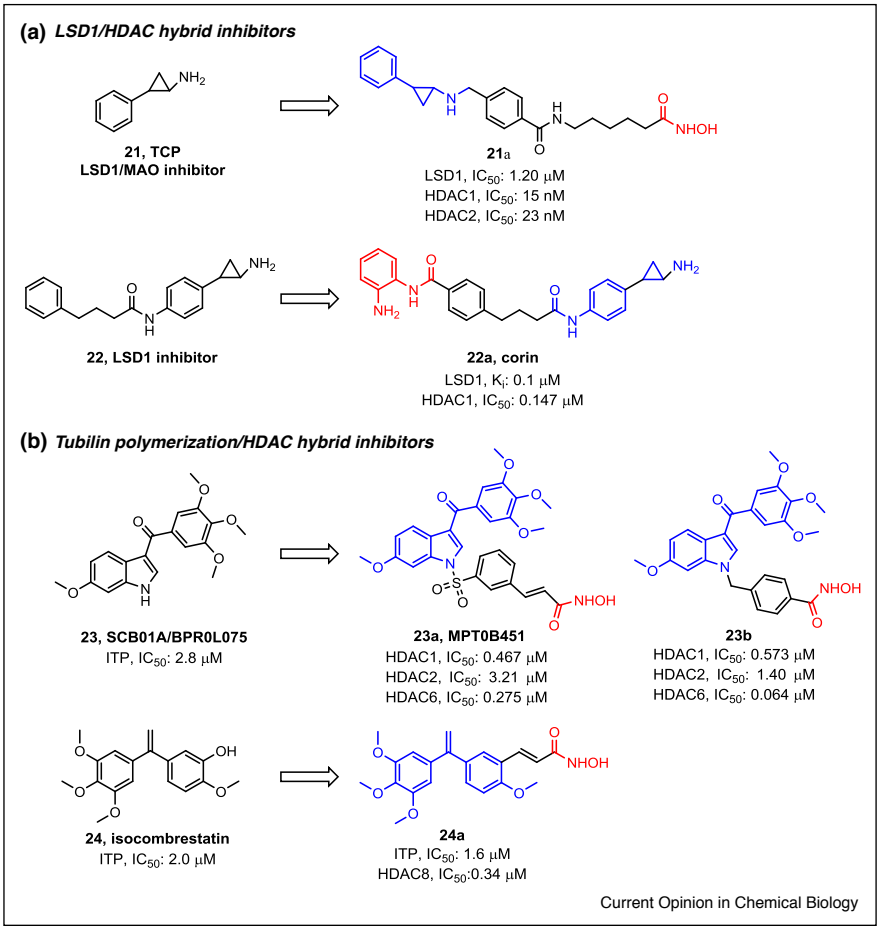
Development of HDAC/kinases hybrid inhibitors **17a–20a**.

activity in the leukemia line MV4-11 (IC_{50} values: 0.047 (**18a**), 1.46 (**17**), and 0.43 (**1**) μM), hepatic carcinoma HuH-7 (IC_{50} values: 0.48 (**18a**), 1.72 (**17**), and 1.97 (**1**) μM), and prostate cancer PC-3 cell lines (IC_{50} values: 1.08 (**18a**), 0.75 (**17**), and 3.07 (**1**) μM). Noteworthy, **18a** (150 mg/kg five-day-on-two-day-off three weeks) inhibited tumor growth in HepG2-xenografted NCr (CrTac: NCr-Foxn1^{nu}) mice more efficiently (95% inhibition) and with lower toxicity than sorafenib (98 mg/kg five-day-on-two-day-off three weeks, 72% inhibition). **18b**, an analogue of **18a**, showed similar results. The mechanism of **18a**, based on PI3K and HDAC inhibition, was confirmed both in cell and animal models, where Akt, P70S6K, and S6 phosphorylation was reduced and H3 and α-tubulin hyperacetylated.

JAK/HDAC hybrid inhibitors

The discovery of a somatic JAK2 mutation (V617F) able to activate the JAK–STAT signaling pathway in most patients with myeloproliferative neoplasms prompted the development of JAK inhibitors, which showed synergistic effects with HDACi [33,34]. These data encouraged Dymock's group to design novel HDAC/JAK2 hybrid inhibitors by merging the structures of a selective JAK2 inhibitor (pacritinib, **19**) with the hydroxamic acid of **1** to achieve the hybrid **19a** [35] (Figure 3). **19a** displayed higher inhibition potency and selectivity for HDAC6 over HDAC1 (about 105-fold) but also over other HDACi. **19a** further afforded almost 10-fold increased potency against HDAC6 compared to **1** and 16-fold against JAK2 compared to **19**. Compound **19a** was tested in a large panel of solid and hematological cancer cell lines, displaying single-digit micromolar IC_{50} values (range 0.94–2.23 μM). Additionally, when tested in non-transformed TAMH cells, **19a** showed lower toxicity (IC_{50}: 8.7 μM). Cell experiments confirmed a dose-dependent induction of H3 acetylation and decreased STAT3 expression in KMS-12-BM, MOLM-14, and HEL92.1.7 cells, along with inhibition of colony formation in HEL92.1.7 cells. The study of molecular mechanisms proved that **19a** acts through JAK–STAT and HDAC pathways inhibition.

Figure 4



Development of HDAC/epi-targets hybrid inhibitors **21a–22a.** Development of HDAC/non epi-targets hybrid inhibitors **23a–24a**.

The same group also designed other JAK2/HDAC hybrids starting from the JAK1/2 inhibitor ruxolitinib (**20**) and **1** [36] (Figure 3). The hybrid with a six-carbon linker (**20a**) revealed the best potency, acting at nanomolar concentrations against both HDAC1/6 and JAK2 and being selective for the JAK family over a panel of 97 different kinases. Additionally, **20a** arrested the proliferation of different solid and hematological tumor cell lines including MDA-MB-231, MCF7, and Jurkat at submicromolar doses (IC_{50} range 0.15–7.36 μM), being slightly less or equally potent than **1**. Compound **20a** impaired the JAK–STAT and HDAC pathways and dose-dependently induced PARP-cleavage, leading to apoptosis. Very recently, Yao *et al.* developed a triple hybrid inhibitor targeting JAK2, HSP90, and HDACs. This inhibitor showed a promising potential in preliminary screening [37]. Other kinase/HDAC dual inhibitors, such as Bcr-Abl/HDAC1 [38] and FGF-receptor-1/HDAC6 inhibitors [39], have been reported.

HDAC and other epi-target hybrids

LSD1/HDAC hybrid inhibitors

Since CoREST1, HDAC1/2, and LSD1 (a histone demethylase) physically interact and cooperate within the CoREST complex [40], the simultaneous blockage of HDAC1/2 and LSD1 has been considered a promising strategy against cancer, leading to combination studies and hybrid compound development [4••] (Figure 4). Duan *et al.* reported novel LSD1/HDACs dual inhibitors [41] wherein the hybrid **21a**, resulting from the combination of tranylcypromine (**21**) and **1**, displayed a non-balanced biochemical activity with nanomolar IC_{50} values against HDAC1/2 and micromolar LSD1 inhibition. Compound **21a** reduced proliferation on a panel of cancer cells (lung A-549, breast MCF-7, gastric MGC-803, and colorectal SW-620) with IC_{50} values ranging from 0.81 to 5.48 μM, being more effective than **1** (IC_{50} range 2.39–8.75 μM). In MGC-803 cells, **21a** increased H3 acetylation (better than SAHA) and H3-Lys4/Lys9

methylation, reduced mitochondrial membrane potential, and induced apoptosis [41]. Recently, combining the pharmacophoric elements of the LSD1 inhibitor **22** with the zinc-binding group of entinostat (**5**), we identified corin (**22a**), targeting the ternary CoREST complex and inhibiting both LSD1 and HDAC1 at submicromolar doses [42••]. In a panel of melanoma cells, **22a** treatment resulted in higher proliferation inhibition than the single agents (**5** or **22**) and their combination. For instance, in WM902B cells, **22a** (1 μM) resulted in 95% of proliferation inhibition versus 80% inhibition by the combination of **5** and **22**. **22a** showed stronger antiproliferative activity than compound **5** or **22** when used alone or in combination also against cutaneous squamous cell carcinoma (MET1, IC_{50} values: 0.006 (**22a**), 0.144 (**5**), 0.171 (**22**), and 0.011 (**5** + **22**) μM; IC1, IC_{50} values: 0.041 (**22a**), 1.03 (**5**), 0.448 (**22**), and 0.294 (**5** + **22**) μM) with up to 7-fold improvement compared to the combined treatment in IC1 cells. The negligible toxicity in primary keratinocytes and melanocytes further highlighted the pharmacological advantage of the hybrids compared to the combination of single-agents. Cell studies confirmed the dual-inhibition mechanism of **22a**, which selectively upregulated a number of tumor-suppressor genes, as well as those involved in differentiation and motility. Showing good pharmacokinetics and safety profile, **22a** (intraperitoneal 30 mg/kg/day) reduced by 61% tumor growth after 28 days without relevant toxicity in melanoma SK-MEL-5 BALB/c mice, while increasing H3-Lys9 acetylation, H3-Lys4 dimethylation, p21, CHOP, and MXD1 expression, and a reducing of Ki67 levels [42••].

HDAC and non-epigenetic targets hybrid inhibitors

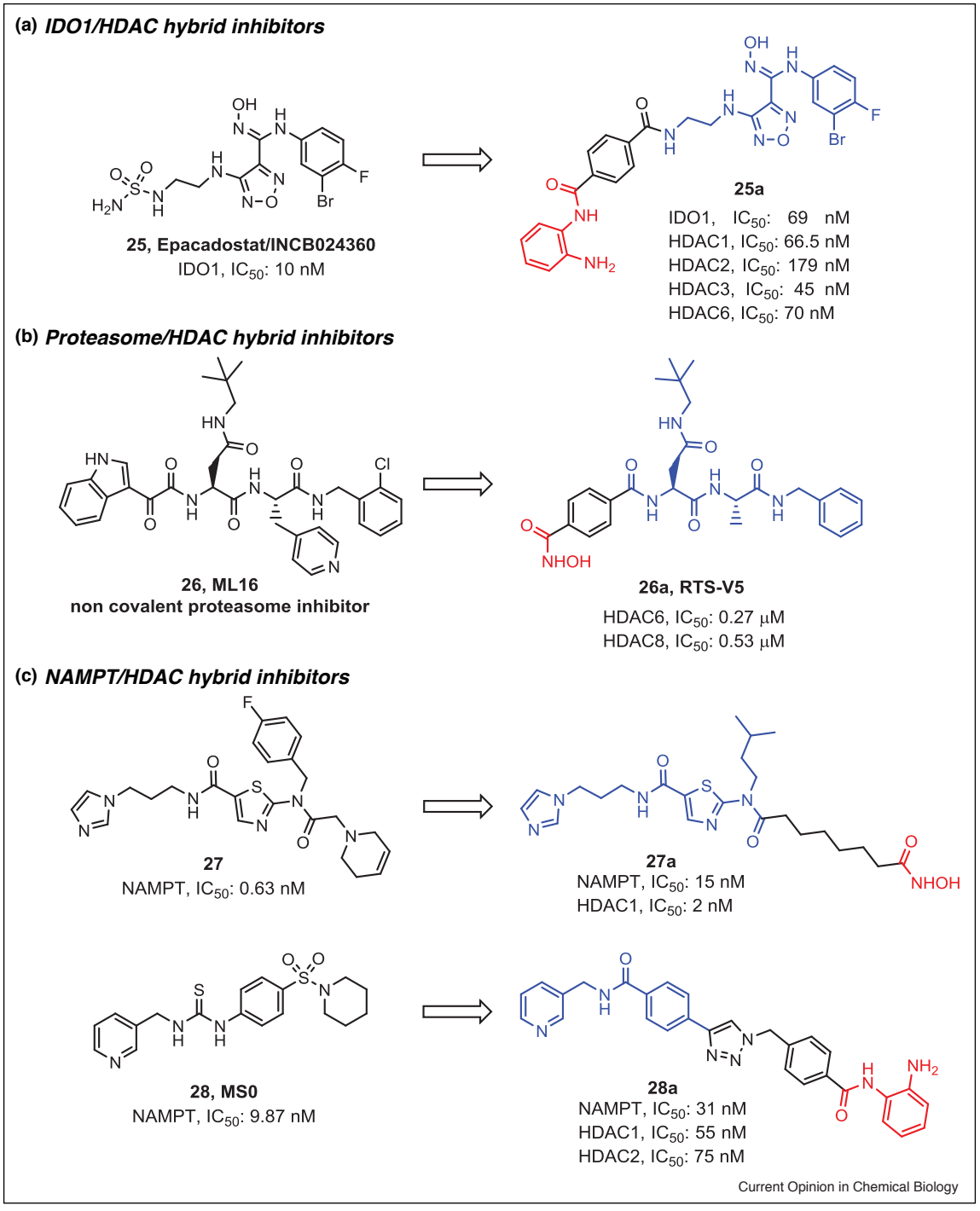
Tubulin polymerization/HDAC hybrid inhibitors

Dual anticancer tubulin polymerization and HDACs inhibitors have been designed starting from colchicine and podophyllotoxin [4••]. The synergistic effect of vincristine and **1** combination in cellular and mice cancer models further validated this strategy [43]. The introduction of different hydroxamic acid-containing or anilide-containing moieties at the N1 position of the clinically studied tubulin-assembly inhibitor SCB01A/BPR0L075 (**23**, NCT01159522) resulted in a new series of dual-targeting agents [44] (Figure 4). Compounds **23a** and **23b** were among the most potent of the series as HDAC (submicromolar IC_{50}s) and tubulin-assembly inhibitors. Both hybrids displayed antiproliferative activity in A549, HCT116, and PC3 cells with IC_{50} values ranging from 0.48 to 0.03 μM. In lung A549 cancer cells, **23a** (IC_{50}: 72.52 nM) displayed 7-fold higher potency than **1** but it was 3-fold less efficient than **23**. Compounds **23a** and **23b** dose-dependently induced H3 and α-tubulin hyperacetylation. Compound **23b** proved a potent inhibitor of tumor growth in PC3 (oral 200 mg/kg day, 20 days, tumor-growth inhibition 68%) and RPMI-8226 (intraperitoneal 100 mg/kg day, 15 days, tumor-growth inhibition 58%) xenografted nude mice without significant toxicity [44]. Compound **23a** arrested HL-60 and PC3 cell cycle in the G2/M and induced apoptosis. In HL60 and PC3 xenografted mice, **23a** dose-dependently suppressed tumor growth (HL60: oral 200 mg/kg day, days, tumor-growth inhibition 54%; PC3: intraperitoneal 100 mg/kg day, 20 days, tumor-growth inhibition 41%) without unspecific toxicity [45]. Putting together the pharmacophoric elements of the tubulin polymerization inhibitor isocombrestatin A-4 (**24**) and of the HDACi belinostat (**3**), Lamaa *et al.* obtained another series of dual-acting agents [46]. Among them, compound **24a** potently inhibited tubulin polymerization, displaying selective HDAC8 inhibition, and produced cytotoxic effects on a panel of cancer cells (K562, PC3, glioblastoma U87, pancreatic BXPC3, colon adenocarcinoma HT-29), being up to 100-fold better than **24** and PCI34051 (an HDAC8 inhibitor). The PC3 cell line was the most responsive (GI_{50} values: 0.4 (**24a**), 2660 (PCI34051), and 3.0 (**24**) nM). Importantly, in the CA-4 refractory human colon adenocarcinoma HT-29 cells, **24a** displayed a lower GI_{50} value (2 nM) than **24** and trichostatin A (HDACi) alone or in combination (GI_{50} values in nM: 276 for **24**, 34 for trichostatin A, 3.8 for **24** + trichostatin A). Compound **24a** arrested cell cycle in the G2/M phase and induced apoptosis by disrupting the microtubule asset, without any relevant toxicity in peripheral blood lymphocytes [46]. More recently, levofloxacin-based hydroxamic acid-containing dual tubulin/HDAC inhibitors have been also developed [47].

IDO1/HDAC hybrid inhibitors

The recent findings on the HDACi-mediated reactivation of anti-tumor immune response [48], fostered their combination with immune checkpoint inhibitors (Figure 5). For instance, indoleamine-2,3-dioxygenase (IDO1) inhibitors proved more efficient in combination with other anticancer agents than alone [49]. In 2018, Fang *et al.* published the first study on the development of dual IDO1/HDAC inhibitors, obtained by the fusion of the iron-binding group of the clinically studied inhibitor epacadostat (**25**) to the typical zinc-binding group of HDACi [50]. Compound **25a** showed balanced and potent inhibition against both IDO1 and HDACs, and micromolar antiproliferative activity against lung LLC and A549, and colon CT-26, HCT-116 and HT-29 cells (IC_{50} ranging from 5.1 to 59.8 μM). Compound **25a** arrested HCT116 cell cycle in the G2/M phase and induced apoptosis. Additionally, it reduced plasma-kynurenine levels *in vivo*, showing a promising anticancer activity in the C57BL16 xenografted immunocompetent murine LLC tumor model. In this mouse model, **25a** (oral 100 mg/kg twice every day) displayed 56% tumor growth inhibition after 14 days, resulting in comparable or better efficacies than those featured by **1** (41%) or **25** (54%) [50].

Figure 5



Development of HDAC/non epi-targets hybrid inhibitors **25a–28a**.

Proteasome/HDAC hybrid inhibitors

Recently, a synergistic anticancer activity has been found by combining HDAC6 and proteasome inhibitors, leading to dual proteasome and aggresome blockage followed by apoptosis. [51] On these bases, the first-in-class dual HDAC-proteasome ligand RTS-V5 (**26a**) was developed by Bathia *et al.* [52••] (Figure 5). The non-covalent proteasome inhibitor ML16 (**26**) and the selective anti-HDAC6 compound **9** were used as a starting point for the new hybrid design. **26a** inhibited both HDAC6/8 and the

chymotrypsin-like proteasome activity in the submicromolar range. However, this hybrid was less potent than the proteasome inhibitor bortezomib. The molecular mechanism of **26a** was studied in SEM cells where it induced apoptosis and autophagy, arrested cell cycle, and colony formation. Moreover, **26a** displayed potent anticancer activity against a panel of chemoresistant leukemic and multiple myeloma cell lines (e.g. SEM, IC_{50} values: 0.89 (**26a**) and 1.61 (ricolinostat, HDAC6 inhibitor) μM; KCL-22, IC_{50} values: 3.14 (**26a**) and 3.75 (ricolinostat) μM), as well as against therapy-refractory primary patient-derived leukemia cells (**26a** IC_{50} range 1.51–5.23 μM; ricolinostat showed comparable potency).

NAMPT/HDAC hybrid inhibitors

The promising anticancer effects of the combination of FK866, a nicotinamide phosphoribosyltransferase (NAMPT) inhibitor, [53] and HDACi promoted the development of dual NAMPT/HDACs inhibitors. Chen *et al.* applied a pharmacophore fusion approach between the thiazolocarboxamide of NAMPT inhibitors (**27**) and the typical moieties of **1** or **5** [54] (Figures 1 and 5). Among the newly synthesized derivatives, **27a** was the most potent with a balanced nanomolar activity against both targets. Against HCT116, A549 and HepG2 cells, **27a** displayed comparable or lower cell proliferation inhibition when compared to **1** or the NAMPTi FK866 (HCT116, IC_{50} values: 30 (**27a**), 9.6 (**1**), and 1.6 (FK866) μM; A549, IC_{50} values: 5.2 (**27a**), 4.4 (**1**), and 3.7 (FK866) μM; HepG2, IC_{50} values: 24 (**27a**), 9.1 (**1**), and 0.89 (FK866) μM). In colon HCT116 cells, **27a** dose-dependently increased H3 and H4 acetylation, while reducing NAD^+ levels with moderate cytotoxicity. When intraperitoneally administered (25 mg/kg 21 days, twice a day) to HCT-116 xenografted nude mice, **27a** displayed increased or similar tumor growth inhibition (42%) values but lower toxicity than **1** (25 mg/kg, tumor growth inhibition 33%) and FK866 (15 mg/kg, tumor growth inhibition 39%) [54]. An iterative structure-based strategy together with structure–activity-relationship investigation led to the identification of compound **28a** as potent and balanced dual NAMPT and HDAC1/2 inhibitor. It displayed comparable or better inhibition of cancer cell proliferation than **1** and was equally or slightly less potent than FK866 (HCT116, IC_{50} values: 2.4 (**28a**), 3.1 (**1**), and 1.6 (FK866) μM; MDA-MB-231, IC_{50} values: 10 (**28a**), >100 (**1**), and 1.3 (FK866) μM; HepG2, IC_{50} values: 4.5 (**28a**), 4.1 (**1**), and 0.89 (FK866) μM). The direct inhibition of NAMPT and HDAC by **28a** was proved also in living cells where it induced apoptosis, autophagy, and arrested HCT116 cell cycle in the G2 phase. In HCT116 xenografted nude mice, **28a** (intraperitoneal 25 mg/kg twice every day) displayed 69% tumor-growth inhibition after 21 days, being more effective than **1** (intraperitoneal 25 mg/kg twice every day, tumor-growth inhibition 33%) and FK866 (intraperitoneal 15 mg/kg twice every day, tumor growth inhibition 39%) [55•].

Other HDAC-based dual hybrids

A number of different dual-acting hybrid compounds, involving HDACs and other epigenetic and non-epigenetic targets, are being developed. They include dual inhibitors targeting HDAC together with the G9a histone methyltransferase [56], DNA methyltransferases (DNMTs) [57], the bromodomain BRD4 [58–60], the poly-ADP-ribose polymerases [61], cyclooxygenases [62], the estrogen receptor α [63], and DNA [64].

Conclusion

In the 'hybrid drug' scenario, HDACs are becoming the pillar for the development of multi-targeting compounds with greater potential in cancer therapy. Indeed, the dual EGFR/HDAC hybrid CUDC-101 (**10a**) and PI3Ks/HDAC hybrid CUDC-907 (**17a**) inhibitors overcame the cancer resistance to receptor tyrosine kinase inhibitors, displayed improved therapeutic effects than the single acting compounds either alone or in combination, and entered the clinical arena, strongly improving the poor efficacy of HDAC inhibitors in solid tumors. The novel hybrids **16a** (HDAC/CDK-4/JAK1i) [25••] and **22a** (LSD1/HDACi) [42••] are also showing a clear correlation between the biochemical, cellular, and animal data. Displaying synergistic effects, such hybrids scored very promising and motivating results in pre-clinical experiments, proving therapeutic improvement compared to the single targeting agents alone or in combination. Nevertheless, the hybrid design process also presents several caveats, limits, and challenges. Among them, retaining the single-targeting compounds' potency against their respective targets and the drug likeness properties with acceptable ADMET profile, while avoiding increased toxicity rates and off-target effects due to drop in target selectivity. The hybrid design and development should be preceded by the validation of the combination between the two single-acting inhibitors. In these preliminary investigations, the most responsive cellular and mouse models should be selected. In this regard, it is unfortunate that several studies did not report on the comparison between the hybrid inhibitors and the combinations of single agents. Therefore, the actual improvement (if any) afforded by the dual inhibitors is often hard to evaluate and validate. Despite these difficulties, the results hereby summarized demonstrate that the development of efficient multi-targeting hybrids represents an avenue that should be further pursued, considering the results reached by CUDC-101 (**10a**) and CUDC-907 (**17a**), and more recently by **16a** and **22a**.

Conflict of interest statement

Nothing declared.

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