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Epigenetic Polypharmacology: A New Frontier for Epi-Drug Discovery

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Abstract

Recently, despite the great success achieved by the so-called “magic bullets” in the treatment of different diseases through a marked and specific interaction with the target of interest, the pharmacological research is moving toward the development of “molecular network active compounds”, embracing the related polypharmacology approach. This strategy was born to overcome the main limitations of the single target therapy leading to a superior therapeutic effect, a decrease of adverse reactions, and a reduction of potential mechanism(s) of drug resistance caused by robustness and redundancy of biological pathways. It has become clear that multifactorial diseases such as cancer, neurological and inflammatory disorders, may require more complex therapeutic approaches hitting a certain biological system as a whole. Concerning epigenetics, the goal of the multi-epi-target approach consists in the development of small molecules able to simultaneously and (often) reversibly bind different specific epi-targets. To date, two dual HDAC/kinase inhibitors (**CUDC-101** and **CUDC-907**) are in advanced stage of clinical trials. In the last years, the growing interest in polypharmacology encouraged the publication of high quality reviews on combination therapy and hybrid molecules. Hence, in order to update the state-of-art of these therapeutic approaches avoiding redundancy, herein we focused only on multiple medication therapies and multi-targeting compounds exploiting epigenetic plus non-epigenetic drugs reported in literature in 2018. In addition, all the multi-epi-target inhibitors known in literature so far, hitting two or more epigenetic targets, have been included.

Keywords

Polypharmacology; epigenetics; hybrid molecules design; cancer

1 INTRODUCTION

1.1 The polypharmacology strategy

Epigenetics (from the Greek words *επί*, over, and *γενετικός*, genetics) studies the heritable and reversible changes in gene expression that cannot be explained by changes in the DNA sequence of bases. These alterations are governed by at least three main

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mechanisms: covalent modifications to the cytosine residues of DNA, histone covalent chemical modifications such as acetylation, methylation, phosphorylation, etc., also known on the whole as chromatin remodeling, and noncoding RNAs. Proteins taking part in chromatin remodeling complexes can add, remove, or read such epigenetic modifications, and are classified as “writers”, “erasers” or “readers”, respectively, determining either transcription or repression of specific genes. Since dysfunctional gene regulation is responsible for many human diseases, the modulation of epigenetic processes is presently considered an innovative and very interesting therapeutic strategy.¹ In the last few years, the research in drug discovery shifted its attention from the “one drug, one target” to the “network active compounds” approach, which is the cornerstone of the modern polypharmacology.² In case of failure of the so-called “magic bullets”, three different and alternative strategies may be followed: the multiple-medication therapy (MMT, drug combination), the multi-compound medication (MCM, which consists in the association of multiple active principles in the same pharmaceutical formulation) or the new, promising multi-target-directed ligands (MTDLs, using single multi-targeting compounds).³ The polypharmacology approach was born with the aim to overcome the limitations of the single-targeted therapy, such as the potential mechanism(s) of resistance caused by redundancy and robustness of biological pathways.⁴ Polypharmacology has the aim to simultaneously hit different targets, all related to the onset and development of a certain disease, although at different levels.⁵ Notably, polypharmacology is quite different from “compound promiscuity”. In fact, the so-called promiscuous (or “dirty”) drugs are typically characterized by a broad spectrum of biological activities directed against targets often not related to the specific disorder of interest, thus leading to a plethora of adverse reactions. Drug promiscuity is often responsible for the occurrence of drug toxicity at the pre-clinical stage.

During the last years, several research groups questioned the possibility to identify structural and not structural features emerging from statistically significant correlation studies (such as lipophilicity, positive or negative charges, basic centers, binding site similarity, flexibility, etc.) common to these promiscuous molecules, in order to identify them as early as possible during the drug discovery process, thus reducing the risk of failure.^{4,6,7} Just to give an example, the clinical application of the anti-allergy drug astemizole⁸ was terminated because, in addition to the desired antagonism of H1 receptors, it inhibits *h*ERG heart potassium channels leading to serious arrhythmia events. For this reason, the dirty drug astemizole was withdrawn from the market.⁹ Despite “promiscuity” is related to negative connotation (off-target interactions), it can be potentially useful in the hands of a medicinal chemist. Medicinal chemists could try to readdress the drug’s unwanted widespread multi-target effects into a specific and desired activity, potentially discovering a new target and allowing drug repurposing.^{7,10} This is the case, for example, of the antifungal agent itraconazole, which exerts its pharmacological effects through inhibition of CYP350-mediated ergosterol synthesis. Follow-up studies showed that itraconazole is also able to inhibit Hedgehog signal transduction and angiogenesis, opening the way for its use as anticancer agent. High doses of itraconazole showed moderate antitumor activity in a phase II clinical trial based on patients affected by castration-resistant prostate cancer ().^{9,11} In cancer, it has become clear that genetic, epigenetic and metabolic factors all contribute to

neoplasia, involving significant changes in molecular networks at the base of many cell functions such as growth, differentiation, development, and death. This is the reason why multi-factorial diseases like cancer may require more complex therapeutic approaches, able to simultaneously interact with various signaling pathways and various cross-talks existing between epigenetic and not epigenetic players. Thus, an effective anticancer therapy should not consider a single pharmacological target, but the biological system as a whole.⁴ Some of the most important challenges of the polypharmacology strategy are the identification of the most appropriate targets and of the relative hit compound(s), as well as the optimization of its (their) structure-activity relationship.

1.2 An overview on single epigenetic targets and related inhibitors in cancer

Recently, a lot of small molecules characterized by high affinity and reversible binding to a specific epigenetic target have been discovered, resulting in seven approved anticancer agents and a number of clinical candidates which are shown in Table 1.

The first epigenetic modulators reaching the FDA approval for the treatment of hematological malignancies were the two DNA methyltransferase (DNMT) inhibitors (DNMTi), azacytidine **1** and decitabine **2**, in 2004 and 2006, respectively.

DNMT is a class of enzymes able to catalyze the transfer of a methyl unit from *S*-adenosylmethionine (SAM), the methyl donor co-substrate, to the C5 of cytosine residues at CpG dinucleotide sequences within DNA. 5-Methylcytosines recruit proteins containing methyl binding domains (MBDs), leading to a global silencing of gene expression, especially due to the contribution of histone deacetylases (HDACs) and/or some histone methyltransferases (HMTs), two families of chromatin remodeling enzymes (see below). These enzymes are able to remove acetyl groups (HDACs) or to add methyl units (HMTs) to histone tails, allowing the recognition and binding of chromatin silencers, and leading to a transcriptionally inactive state of chromatin (heterochromatin).⁶ While in physiological conditions the cytosine methylation is concentrated in non-coding regions of DNA, in cancer cells features an increased methylation at the promoter regions of genes encoding for tumor suppressor factors or other proteins with anti-proliferative effects, joined to a global DNA hypomethylation. The potential utility of DNMTi in the anticancer therapy consists in their ability to reactivate silenced tumor suppressor genes and pathways.^{7,8} In humans, two kind of DNMTs are known, DNMT1 and DNMT3, while DNMT2 has been recognized as a RNA methyltransferase.¹² DNMT1 is the most abundant, and is able to catalyze the methylation of hemi-methylated DNA maintaining the methylation status during cell replication. DNMT3 (including three isoforms: DNMT3A, -3B and -3L, the last catalytically inactive) is defined as “*de novo*” methyltransferase, acting on both hemi-methylated and non-methylated DNA.^{13,14}

Azacytidine **1** and decitabine **2** (Table 1), characterized by a 1,3,5-triazine ring, are two approved drugs for the treatment of myelodysplastic syndrome (MDS), that often progresses to acute myeloid leukemia (AML). Through a triple phosphorylation, these pro-drugs are metabolically transformed into the active species which are incorporated into DNA and accepted as DNMT substrates. Nevertheless, after the nucleophilic attack of the enzyme at the triazine C6 position and the introduction of the methyl group at N5 from the SAM co-

substrate, they do not allow the final elimination step as a result of the lack of a proton at the N5 position, leaving the enzyme irreversibly and covalently inhibited.¹⁵ However, despite their high efficacy, such drugs suffer from low selectivity, poor bioavailability, chemical instability and toxic side-effects. Non-nucleoside DNMTi are objects of great interest for the researchers since they do not need to be incorporated into DNA, overcoming the problem of toxicity linked to the use of nucleoside analogues. Unfortunately, many of them display low potency, scarce target selectivity, and unknown mechanisms of inhibition.

Among histone modifications, (de)acetylation and (de)methylation of lysine residues are the most studied. The acetylation reaction is catalyzed by histone acetyltransferases (HATs) using acetyl-CoA as co-substrate. Lysine acetylation decreases the affinity of histones for DNA, allowing the passage from the most compact heterochromatin to the more relaxed euchromatin form. This allows the recruitment of transcription factors, chromatin remodeling complexes and, finally, gene transcription. HDAC enzymes, instead, restore the free amine state of lysines leading to closure of chromatin and gene silencing. Thus, HDAC inhibitors (HDACi) are epi-drugs able to reactivate signaling pathways silenced by deacetylation, in cancer as well as in non-cancer diseases. To date, 11 zinc-dependent human HDAC isoforms have been classified into three classes (I, II and IV), differing in structure, enzymatic function, sub-cellular localization, and tissue expression. In addition to these “classical” HDACs, the mammalian genome encodes another group of deacetylases, known as sirtuins (class III HDACs), which hydrolyze acyl-lysines through a different mechanism involving nicotinamide adenine dinucleotide (NAD⁺) as co-substrate. Importantly, the HATs/HDACs have also thousands of non-histone protein substrates.¹⁶ A pharmacophoric model for HDACi has been described including i) a cap group binding at the enzyme surface and partially exposed to the solvent, ii) a spacer mimicking the lysine side-chain of the substrate, and iii) a moiety able to complex the zinc ion, crucial for the HDAC catalytic action.¹⁷

Vorinostat **3** (SAHA, suberoylanilide hydroxamic acid, Table 1) was the first pan-HDAC inhibitor (not selective for any HDAC isoform) approved by FDA in 2006 for the treatment of refractory cutaneous T-cell lymphoma (CTCL). It is characterized by an anilide cap group, a linear spacer of six methylene units and a hydroxamate function as zinc ion binder. Romidepsin **4** (FK-228, Table 1) is a natural prodrug approved by FDA in 2009 for the treatment of refractory CTCL, carrying a disulfide bridge that is reduced *in vivo* to release the thiol zinc binding moiety. Romidepsin **4** shows mainly inhibitory activity against class I HDACs rather than pan-inhibition. Belinostat **5** and panobinostat **6** (Table 1) are two other hydroxamate-containing pan-HDAC inhibitors approved by FDA, the first in 2014 for the treatment of refractory peripheral T-cell lymphoma (PTCL), the latter in 2015 for the treatment of refractory or relapsed multiple myeloma (MM). Tucidinostat **7** (chidamide, Table 1) is the first benzamide-type HDACi approved for clinical use. It inhibits HDAC1/2/3/10 and was approved by the Chinese FDA in 2014 for the treatment of PTCL. Sodium valproate (VPA) **8** (Table 1) is a known antiepileptic drug belonging to the short-chain fatty acid series of HDACi. It selectively inhibits class I HDACs and reduces tumor growth and metastasis formation in various animal models. Entinostat **9** and mocetinostat **10** (Table 1) are two benzamide-containing, class I-selective HDACi currently in clinical trials

for the treatment of numerous solid tumors. Abexinostat **11**, pracinostat **12**, quisinostat **13**, resminostat **14**, givinostat **15** and rocilinostat **16** (Table 1) are examples of hydroxamates pan-HDACi (with the exception of **16**, quite selective for HDAC6) in clinical trials for the treatment of several hematological (**11-13**, **15** and **16**) and solid tumors (**14**). Among them, pracinostat **12**, resminostat **14** and givinostat **15** granted the status of orphan drugs for AML, hepatocellular carcinoma, and Duchenne muscular dystrophy, respectively.¹⁸⁻²⁰ Nicotinamide **17** is the only sirtuin inhibitor currently used in clinics for the treatment of solid tumors (Table 1).

Recently, clinical candidates have been obtained for other epigenetic targets such as lysine methyltransferases (KMTs), arginine methyltransferases (PRMTs), lysine demethylases (KDM) and bromodomains (BRDs). KMTs constitute a large family of enzymes able to catalyze the transfer of one, two and/or three methyl groups to lysine residues using SAM as the methyl donor co-substrate. Similarly, PRMTs perform methylation (single or double, the latter symmetric or asymmetric) at arginine residues of histone and non-histone proteins.^{21,22}

Differently from DNA methylation, lysine methylation can lead to either transcriptional activation or repression, depending on the specific lysine residue modified, and on the extent of methylation (me1, me2, or me3). GSK2816126 **18**, tazemetostat **19** and CPI-1205 **20** (Table 1) are selective, catalytic inhibitors of both wild type (wt) and mutant forms of the methyltransferase EZH2 (enhancer of zeste homolog 2), currently in clinical trials in patients with various lymphomas, multiple myeloma, and solid tumors.^{23,24} Pinometostat **21** (Table 1) is a picomolar inhibitor of the H3K79 methyltransferase DOT1L (disruptor of telomeric silencing 1-like), with more than 30,000-fold selectivity against other KMTs. When used in rearranged-MLL (mixed-lineage-leukemia) cells and xenograft models, **21** reduced H3K79 methylation level, decreased *MLL* target gene expression, and induced selective leukemia cell death.^{25,26}

JNJ-64619178 **22**²⁷ and GSK3326595 **23**²⁸ (Table 1) are two potent and selective PRMT5 inhibitors that induced tumor regression in solid cancers as well as in hematologic malignancies, supporting clinical testing in patients with these kinds of cancer.

To date, two families of KDMs have been identified.^{27,29} The first is the KDM1 family, including LSD1 and LSD2 (lysine-specific histone demethylase 1 and 2), able to remove methyl units through an oxidative amination process using flavin adenine dinucleotide (FAD) as cofactor. The second KDM family, containing KDM2-7, is known as Jumonji C (Jmj-C) domain-containing protein family and uses an α -ketoglutarate/Fe(II) ion-dependent mechanism to catalyze the hydroxylation of a lysine *N*-methyl group, followed by its release as formaldehyde. It is known that increased activity or expression of members of both the KDM families are implicated in different types of cancer. Given the similarity of the catalytic mechanism of LSD1 and monoamine oxidases (MAOs), MAO inhibitors (MAOi) were investigated for their potential ability to inhibit LSD1. While the majority of MAOi failed to inhibit LSD1, tranilcypromine **24** (Table 1) is able to covalently inhibit LSD1 in the micromolar range, via radical species that originate from the opening of the cyclopropane ring.³⁰ Two tranilcypromine analogs, ORY-1001 **25** and GSK2879552 **26**

(Table 1), showed potency in the nanomolar range and high selectivity for LSD1 over MAOs, and entered clinical trials for the treatment of leukemia and small cell lung carcinoma.^{31,32}

BRDs are characterized by a hydrophobic pocket that specifically recognizes the acetyl mark of acetylated lysine residues. To date, 61 different BRDs are known, including the BET (bromo- and extra-terminal) domain comprising BRD2, BRD3, BRD4, and BRDT. An enrichment of BRD4 in enhancer regions is connected to the expression of oncogenes in cancers, while translocations fusing BRD3 or BRD4 to the NUT oncogene are responsible for NUT midline carcinoma, a type of cancer with a very low life expectancy. The BET inhibitors CPI-0610 **27**, GSK525762 **28** and OTX015 **29** (Table 1) are being tested in clinical trials for a variety of cancers including multiple myeloma (**27**), NUT midline carcinoma (**28**), acute leukemia and triple negative breast cancer (**29**).^{33,34}

One of the main reasons for which a single epi-target approach can be ineffective is the emergence of pharmacological resistance. Phenotypic aberrations at the base of neoplastic diseases are the result of a complicated signaling network system that evolves and changes over time to evade drug-induced events such as cell death, growth inhibition, DNA repair, and metabolic alterations. The goal of drug discovery in the epi-drug research is no longer simply the design of highly potent and selective molecules acting against a known epi-target, but is moving towards the identification of network-active compounds with multi-targeting properties. This latter strategy could be obtained by using the MTDLs approach.⁴

2. Multiple-Medication Therapy (MMT), Multi Compound Medication (MCM), and Multi-Target-Directed Ligands (MTDLs) approaches

The theoretical concept at the base of polypharmacology is to achieve an improved therapeutic effect hitting different targets, all responsible for the onset of a specific complex disease. All the factors and their relative interactions characterizing a molecular pathway could be considered as potential targets for a polypharmacology-based therapy. In fact, multi-target drugs can hit a cellular pathway at different levels with the goal to get a synergistic effect. This goal can be achieved by all the three aforementioned approaches that, despite having the same purpose, show marked differences. While the MMT and the MCM strategies are based on the combination/association of two or more active principles, each with its own specific target, the MTDLs strategy is focused on the development of only one molecule able to simultaneously interact with different targets.

The MMT could increase or maintain the desired therapeutic efficacy potentially requiring a lower dose of each drug, thus minimizing drug resistance and adverse reactions compared to single-drug treatment.³⁵ One of the main disadvantages of the application of the so-called “drug cocktails” is the negative impact on patient compliance, that could be compensated by the MCM approach, which takes advantage of “polyvalent-pills” whereby two or more agents are co-formulated in a single tablet.³⁶ However, both approaches show potential pharmacodynamic (PD) and pharmacokinetic (PK) limitations, such as drug-drug interactions and the possible different solubility of the active substances that may modify the bloodstream uptake, not guaranteeing the simultaneous presence of the required drugs at the

desired concentrations in the target tissue. In addition, when the agents are used in combination or association, regulatory agencies generally require the demonstration of safety of each individual drug before clinical trials. If the drugs of interest are owned by different pharmaceutical companies, this procedure can require an extended time.^{2,37}

Despite the common hallmarks of cancer, each tumor, as well as the therapeutic outcome of each patient, is different from the others. One of the central aspects of MMT consists in the possibility to choose different dosages, establishing a prolonged administration of drugs and increasing the number and type of potential therapeutic schedules, paving the way to a personalized treatment. The MMT approach allows to modulate the dose of each single drug within the cocktail (if necessary), according to the patient's needs.^{4,38–40} In light of these evidences, we can compare the MMT and MTDL approach uncovering their strengths and weaknesses. To date, despite the MTDL overcame some of the typical limitations of the MMT and MCM strategies, with in principle better results at both PK and PD level (e.g. better patient compliance, no solubility/biodistribution issues, greater efficacy, lower toxicity, etc.), the MMT still proves easier to apply and customize, due to the virtually infinite drug combination and dosage possibilities.^{4,40}

3. The MMT approach

Epigenetic modulators, such as pan-HDAC or DNMT inhibitors, showed efficacy in the treatment of different kinds of tumors such as ovarian cancer,⁴¹ CTCL,⁴² breast cancer,⁴³ and MDS.⁴⁴ To date, the combination therapy is more extensively explored in the clinic than the use of multi-targeting single drugs. The first clinical success of the MMT approach dates back to 1980 and consists in the treatment of childhood acute lymphoblastic leukemia (ALL), based on the co-administration of methotrexate **30**, vincristine **31**, prednisone **32** and 6-mercaptopurine **33** (Figure 1).⁴⁵

Regarding epi-drugs, it has been recently highlighted their capability (mostly HDACi and DNMTi)^{46,47} to increase chromatin accessibility to conventional DNA-damaging chemotherapeutic agents, markedly enhancing their effects and establishing a rationale for their combination. Several pieces of evidence showed that the CpG island methylation is responsible for the induction of drug resistance, and this effect can be reversed through the treatment with hypomethylating agents^{48–51} including zebularine⁵¹ and decitabine **2**.^{51,52} The combination of HDACi with cytostatic agents (such as doxorubicin and paclitaxel) has been widely evaluated in both preclinical and clinical contexts. Here, a reliable increase in the therapeutic outcome was achieved, due to the downregulation of proangiogenic and tumorigenic factors (i.e. HIF-1 α and VEGF),^{53,54} the repression of DNA repair systems⁵⁵ as well as the enhancement of apoptosis induction.^{55–57} Furthermore, HDACi can also induce immune potentiation responses by increasing the anticancer activity of IL-2.⁵⁸ The most commonly evaluated epigenetic combinations are those based on HDAC/DNMT inhibitors, since DNMTi incorporation into DNA is restricted to cell cycling cells.⁵⁹ In addition, this combination could be useful due to the induction of synergic effects in the modulation of gene expression, in particular for *IRF4*, *MYC* and several related genes. It is important to underline that the downregulation of these genes was not identified after either HDACi or DNMTi treatment alone.^{60,61} A lot of preclinical evidences collected in AML or MDS cell

lines or in *ex vivo* cultured patient cells, such as improved cell growth arrest, loss of clonogenic potential and DNA synthesis inhibition, support the combination of HDACi with the nucleoside DNMTi azacytidine **1** or decitabine **2**.^{62–64} HDACi were able to sensitize cancer cells to cellular stress-based radiotherapy and hypothermia treatment through induction of apoptosis and arrest of protective cell mechanisms.^{65,66} The combination of modulators of the histone acetylation and DNA methylation status could be useful also to counteract the onset of cancer resistance.⁶⁷

3.1 New preclinical studies (2018) based on epigenetic modulators in combined anticancer therapy

Combination therapy based on decitabine **2** and vorinostat **3** (combination A, Figure 2) has been recently evaluated against pancreatic cancer by Han *et al.*,⁶⁸ demonstrating improved effects *in vitro* when compared to the single drug application. Treatment with vorinostat **3** or decitabine **2** alone induced pancreatic cancer cells cycle arrest, as well as inhibition of migration and proliferation. Importantly, while vorinostat **3** induced cell apoptosis also as monotherapy, decitabine **2** alone was unable to cause this effect. Combination A led to stronger effect compared to each epigenetic agent alone, resulting more effective in proliferation and migration inhibition, G₂ cell cycle arrest, apoptosis induction, and in enhancing tumor suppressor genes (*P16* and *TP53*) expression levels. Han *et al.* further reinforced this evidence postulating the inhibition, by both drugs, of the PI3K/AKT/PTEN signaling pathway.⁶⁸

Malignant pleural mesothelioma (MPM) is a rare and strongly aggressive cancer, and many shreds of evidence highlighted that it is mostly associated with asbestos exposure.⁶⁹ Unfortunately, none of the available treatments is effective, due to developed resistance to all conventional therapies. However, recently epigenetics has been claimed to play a role in this cancer, suggesting the use of epi-drugs for MPM treatment. As a proof of evidence, global hypomethylation and specific promoter hypermethylation (including those of tumor suppressor genes) joined to a decrease in the acetylation status of histone H3 and H4 have been observed.^{70–73}

In previous works focused on a mouse model of MPM, either decitabine **2** or HDACi (sodium valproate **8** or vorinostat **3**) monotherapy proved to be non-effective exerting only weak anticancer activity, while their combination induced antitumor immune response through the expression of tumor anti-antigen factors, especially cancer testis antigen (CTA).^{74,75}

Starting from the adverse reactions (first of all, hematological toxicity)⁷⁶ correlated to HDACi, Bensaid *et al.*,⁷⁷ in order to identify new suitable HDACi for clinical use, tested four recently developed HDACi,^{78,79} two hydroxamates (ODH and NODH **34**, Figure 2) and two benzamides (ODB and NODB), comparing their toxicity and anticancer effect against MPM with those of vorinostat **3** and sodium valproate **8**. The same authors also studied the immunogenic capability of the novel HDACi in combination with decitabine **2**, also measuring the mRNA expression of PD-L1 (programmed death-ligand 1), CTA, RIG-I

(retinoic-acid-inducible protein 1) and MDA5 (melanoma-differentiation-associated gene 5) in MPM cells.

All the tested HDACi were toxic for immune cells, being responsible for the death of 100% CD8 T-cells clones on total lymphocytes at high doses. The combination of the new HDACi NODH **34** and decitabine **2** (Combination B, Figure 2) seemed to be the most promising. In fact, NODH **34** was 100-fold more effective than vorinostat **3** in enhancing histone H3 acetylation in cells, being in addition less toxic. Since this combination induced PD-L1 expression, also the association with anti-PD-L1 agents should be tested with the aim to improve the anti-tumor immune response.⁷⁷

Compared to other breast cancer subtypes, the triple negative breast cancer (TNBC) is the most aggressive as well as the most difficult to treat. Recently, Merino *et al.*⁸⁰ reported a triple combination based on all-trans retinoic acid **35** (ATRA), entinostat **9** and doxorubicin **36** (Combination C, Figure 2), that resulted able to induce differentiation and cell death as well as a significant regression of tumor in TNBC xenograft models. The combination of doxorubicin **36** and entinostat **9** induced the downregulation of cell cycle genes (such *E2F*, *G2M* and *MYC*) and the upregulation of immune checkpoint agonists and cancer testis antigens as well as interferon genes. Moreover, such combination was responsible for an altered expression of genes related to inflammation, differentiation and growth arrest, through topoisomerase II- β inhibition (by doxorubicin **36**) and decreased expression (by entinostat **9**).⁸¹ In particular, entinostat **9** sensitized the cells to the doxorubicin **36** treatment, enhancing G₂ cell cycle arrest⁸² and inducing a more effective up-regulation of cytokines, costimulatory molecules, other inflammatory genes, and tumor antigens with a consequently increased interferon response. The single drug treatment with ATRA **35** led to breast cancer cell death,⁸³ differentiation^{84,85} and inflammation.⁸⁶ However, its limited success in solid tumors⁸⁷ could be due to the frequent epigenetic silencing of the retinoic acid receptor beta (RAR- β).^{88,89} Different studies evidenced that RAR- β is epigenetically silenced and under-expressed in breast cancer, while the treatment with HDACi typically induces RAR- β re-expression, sensitizing the cells to ATRA treatment.^{90,91} The triple combination described above, in addition to the effects observed with the doxorubicin **36**/entinostat **9** combination, markedly induced cancer stem cell differentiation, cell death and necrosis,⁸¹ and increased the expression of different genes playing important roles in the inflammation process, such as *DHRS3*, *IL-1 β* , *CCL26*⁹², *ELF3*,^{93,94} *IL-1 α* , *TNF- α* and *CD14*.

In TNBC patients, the expression of epithelial-mesenchymal transition (EMT) markers and cancer stem cells with CD44⁺CD24^{-/low} phenotype are associated with a poor outcome.^{95,96} Several pieces of evidence support the involvement of epigenetic mechanisms in the activation of EMT and in particular in the modulation of E-cadherin expression. The hypermethylation of the CDH1 promoter plays an essential role in EMT regulation. The E-cadherin expression is also repressed by different EMT inducing factors such as *ZEB1*, *ZEB2*, *TWIST*, *SNAIL* and *SLUG*,^{97–100} and this process is regulated by HDACs,^{98,101–104} establishing a rationale for the application of an epigenetic combination therapy for TNBC treatment.¹⁰⁵ Exploring EMT modulation in their unique TNBC cell model,¹⁰⁶ Su *et al.*¹⁰⁷ decided to test six HDACi (vorinostat **3**, entinostat **9**, SB939, LBH589, quisinostat **13**, tubastatin A) and three DNMTi (azacytidine **1**, decitabine **2** and SGI-110 **37**). These

epigenetic modulators were first tested alone in MTT, migration and invasion, three dimensional culture, and colony formation assays. Once selected as the most potent of them, entinostat **9** and SGI-110 **37** were used in combination to reprogram TNBC cells, attenuating the EMT process by E-cadherin induction in mesenchymal-like cancer cells. The combination of entinostat **9** and SGI-110 **37** (Combination D, Figure 2) showed improved results in cancer cells motility, colony formation, proliferation, apoptosis, and stemness when compared to the use of single agents alone. From a pharmacological point of view, the combination D synergistically suppressed EpCAM cleavage and Wnt signaling, counteracted EMT, reduced the expression of mutant p53, EZH2 and ZEB1, and induced apoptosis as well as H3 hyperacetylation in TNBC.¹⁰⁷ *In vivo* studies showed that entinostat **9** alone, as well as the **9/37** combination, decreased XtMCF xenograft tumor weight in CB17/SCID mice of about 52%. Differently, in NOD/SCID mice the combination D (at higher doses, but with a shorter treatment) reduced tumor weight by 33% compared to the control, while the single target inhibitors **9** and **37** administered alone did not produce significant effects on tumor growth.¹⁰⁷

Regular consumption of bioactive dietary compounds may have the capability to modulate different epigenetic mechanisms, thus playing also an active role in cancer prevention and therapy.^{108–111} Genistein **38** is an isoflavone naturally contained in soybean, able to inhibit DNMT and HDAC enzymes and showing anticancer properties in cells.^{112–115} Recently, genistein **38** has been reported to: i) arrest breast cancer cells cycle in G₂/M phase inducing the expression of *p21^{WAF1/CIP1}*;¹¹⁶ ii) re-sensitize ER-negative breast cancer cells to estrogen-targeted chemotherapy through the activation of estrogen receptor (ER);¹¹⁷ iii) increase the acetyl-H3 and acetyl-H4 levels as well as dimethyl- and trimethyl-H3K4 at the promoter region of the *BTG3* gene in renal cancer;¹¹⁴ iv) increase the acetylation status of H3K4 at the *p21^{WAF1/CIP1}* and *p16^{INK4a}* transcription start sites, mediated by induction of HATs in prostate cancer.¹¹⁸

Sulforaphane **39**, abundant in kale and broccoli sprouts, is a HDACi^{112,113,119} effective against breast cancer stem cells both *in vitro* and *in vivo* suppressing the Wnt/ β -catenin pathway.^{76,120} In addition, Meeran *et al.* showed that sulforaphane **39** induced *hTERT* repression in breast cancer¹²¹ with chemo preventive effects through the induction of cell cycle arrest *via* Kruppel like factor (KLF4) transcription mediation.¹²²

Previous studies conducted by Meeran *et al.* revealed that a diet containing compounds with HDAC and DNMT inhibitory activities altered the epigenome, resulting in the modulation of various aberrant epigenetics patterns in tumor-related genes.^{76,121,123} Therefore, Paul *et al.* decided to evaluate the effects deriving from the combination of genistein **38** and sulforaphane **39** (Combination E, Figure 3).¹²⁴ Genistein **38**/sulforaphane **39** combination decreased the HDAC2 and HDAC3 expression both at protein and mRNA levels, downregulating KLF4, essential in stem cells formation, as well as *hTERT* that is known to be activated when KLF4 binds its promoter region. Combination E resulted more effective than the single agents alone in decreasing cell viability, lowering colony formation and inducing apoptosis in breast cancer cell lines (MCF-7 and MDA-MB-231).

The aim of the study conducted by Paul *et al.*¹²⁴ was to evaluate the *in vivo* anticancer effect of **38** and **39** taking advantage of a diet based on food rich in these two bioactive compounds. Concerning genistein **38**, the authors could not use soy feeding, because it contains many other active compounds, making it difficult to dissect the role of **38**. Thus, the pure **38** was used for the study. Differently, broccoli sprouts contain a 50-fold higher concentration of glucoraphanin, which is converted to the isothiocyanate form (sulforaphane **39**) by myrosinase, thus they can be used as **39** source.^{125,126} Combinatorial **38**/broccoli sprouts dietary regimen, at a physiologically accessible concentration by daily consumption,^{54,127} was orally administered to an ER(–) transgenic breast cancer mouse model and compared with the two bioactive compounds alone. *In vivo* results supported the *in vitro* evidence, since the combination E was more effective than the single drug treatment in reducing tumor size and volume and in extending tumor latency. Future plans are focused on the evaluation of this combination in other kind of cancers such as ER(+) and BRCA1 mutated TNBC.¹²⁴

The combination of platinum-based compounds/HDACi against non-small cell lung carcinoma (NSCLC) founded its rationale mainly in two findings: i) HDACs play a main role in resistance and adaptation to genotoxic treatment in different ways;^{128–135} ii) platinum chemotherapy is the first choice for the treatment of such tumors.^{136–138} A dose escalation phase I clinical study was conducted for panobinostat **6**/etoposide/cisplatin triple combination. However, since a dose-limiting febrile neutropenia and thrombocytopenia was observed at the first dose level of panobinostat **6** (10 mg orally three times weekly on a 2 week on/1 week off schedule), it was not possible to determine a phase II starting dose.¹³⁹ The unacceptable toxicity was probably due to the overlap of adverse reactions induced by each drug, suggesting the potential evaluation of new safer combination therapies. Despite the choice of carboplatin **40** is favored in terms of toxicity, its lower potency requires the combination with other drugs in order to increase its anticancer activity. Recently, Wang *et al.*¹⁴⁰ proposed to combine carboplatin **40** with panobinostat **6** getting a strong improvement in cell proliferation reduction with IC₅₀ values ranging between 4–21 nM in different NSCLC cell lines such as H460, H226, A549, Calu-1 and SKMES-1. These promising outcomes are likely the result of the pleiotropic effects of panobinostat **6** which, in addition to the modulation of histone acetylation, has also hyperacetylating effects on non-histone substrates including proteins that play a fundamental role in carcinogenesis such as STAT3, HSP90, p53, E2F, NF-κB, c-Myc and HIF-1α.^{136–138,141} Most importantly, the hyperacetylation mediated by panobinostat **6** at HSP90 level restores the physiological degradation of different oncoproteins, including epidermal growth factor receptor (EGFR) and its downstream transducers.¹⁴² In addition, it has been shown that HDACi reverse the repression of miRNAs facilitating the degradation of EGFR mRNA,^{143,144} suggesting a potential synergistic effect between panobinostat **6** and EGFR inhibitors against EGFR-mutant NSCLC.^{145,146} Tumorigenic mouse models also confirm the chemo sensitizer effect induced by panobinostat **6**. Indeed, the carboplatin **40**/panobinostat **6** combination (Combination F, Figure 3) led to a markedly stalled disease progression by 92% compared to the negative controls, that also resulted greater than that caused by carboplatin **40** (28%) or panobinostat **6** (54%) alone.¹⁴⁰

To date, only a few effective therapeutic options for the treatment of NRAS-driven melanoma are available.¹⁴⁷ A lot of clinical studies have been conducted focusing on RAS effectors, in particular on inhibitors of phosphatidylinositol-3 kinase (PI3K) signaling pathways¹⁴⁸ and of mitogen-activated protein kinase (MAPK) such as MEK inhibitors,¹⁴⁸ but their therapeutic potential as single agents was modest. Despite the introduction of new therapeutic approaches for the treatment of BRAF-mutant melanoma (first of all immunotherapy treatment),¹⁴⁹ the acquisition of resistance is a significant problem and, in particular, NRAS-mutant (NRAS^{Mut}) melanoma treatment is one of the hardest challenge in this field.^{150–154} Echevarría-Vargas *et al.*¹⁵⁵ demonstrated that BET proteins are overexpressed in NRAS^{Mut} melanoma and, in particular, increased levels of BRD4 mRNA are associated with poor patient survival. Starting from these shreds of evidence, they evaluated the potential of the combination of BETi (OTX-015 **29** or JQ1) and MEK inhibitors (the FDA-approved trametinib **41** or PD901, in phase II clinical trial) restraining NRAS^{Mut} melanoma and offsetting drug resistance (Figure 3). The dual targeting of BET/MEK synergistically prolonged NRAS^{Mut} tumor-bearing mice survival without apparent toxicity, downregulated the expression of the transcription factor TCF19, which is essential for melanoma cells survival, sensitizing it to apoptosis, and mitigated a transcriptional signature associated with innate resistance to already used inhibitors and immune checkpoint agents. The treatment with the BETi/MEKi combination showed the strongest efficacy and tolerability in patient-derived xenograft and immunocompetent syngeneic mouse models. While BETi alone induced mainly cytostatic effects in NRAS^{Mut} melanoma cells *in vitro*, minimal effects have been registered in *in vivo* models. However, the combination of BETi/MEKi was able to induce robust anti-tumor effects in NRAS-mutant melanoma. Considering that the MEK inhibitor trametinib **41** is a FDA-approved drug for BRAF-mutant melanoma patients, and different BETi such as OTX-015 **29** are in advanced clinical trials, clinical studies based on the combination of trametinib **41** and OTX-015 **29** (Combination G, Figure 3) could be rapidly implemented for therapy-resistant melanoma.¹⁵⁵

A panel of HDACi (vorinostat **3** and romidepsin **4**) and BETi (OTX015 **29**, JQ1, I-BET762 and CPI-0610 **27**) were selected by Zhao *et al.*¹⁵⁶ to evaluate the effectiveness of their combination for the treatment of CTCL. The combinations resulted effective since induced apoptotic events, in addition to G₀/G₁ cell cycle arrest as well as reduction of the expression levels of fundamental cancer players such as cyclin D1, NF-κB, c-Myc and IL-15Rα. In particular, the combination based on OTX015 **29** (125 nM) and romidepsin **4** (1 nM) (Combination H, Figure 3) showed the greatest apoptotic effect (60–80%, while OTX015 **29** or romidepsin **4** alone induced only 6% or 31% apoptosis, respectively) at 96 hours of treatment, resulting the most promising combination for clinical use. *Ex vivo* studies on leukemic CTCL cells obtained from patients with Sezary syndrome resulted in higher levels of apoptosis (about 60–90%) following the combined treatment rather than single agents administration. During the use of Combination H, normal CD4⁺ T cells were minimally affected (10%).

Recently, other preclinical studies based on the simultaneous administration of BETi and HDACi in melanoma cells as well as in murine models of pancreatic ductal adenocarcinoma

have been described recently.^{175,157–159} In 2018, the combination of the BETi JQ1 **42** and romidepsin **4** (Combination I, Figure 3) in urothelial carcinoma (UC) inhibited cell cycle progression, suppressed clonogenic growth, and induced apoptosis. In the same system, the treatment with JQ1 **42** alone was able to induce cell cycle arrest but only limited apoptosis. At a molecular level, in UC cells the JQ1 **42**/romidepsin **4** combination increased H3K27 acetylation but decreased H3K4 trimethylation, downregulating anti-apoptotic and oncogenic factors such as survivin, BCL-2, BCL-XL, c-MYC, EZH2 and SKP2 and diminishing AKT phosphorylation.¹⁷⁴

Preclinical studies conducted by Kim *et al.* highlighted the potential of the therapeutic combination between BETi and HDACi or inhibitors of the antiapoptotic protein B-cell lymphoma 2 (BCL2i) in the treatment of advanced CTCL. BCL2 was previously suggested as a targetable pathway on the basis of the common gene alterations that increase BCL2 activity and dependence, including *TP53* deletion, *STAT3* and *STAT5B* amplification, and *CTLA4* deletions.^{160–164} To date, CTCL is generally considered incurable. Indeed, the overall response rates to single agent systemic therapies (such as the retinoid bexarotene or HDACi) range between 20–45% and relapses are common.^{165,166} The MYC oncogene is often amplified in CTCL condition, and the capability of BETi to repress its expression has been widely discussed in literature, in particular for AML, multiple myeloma (MM) and Burkitt's lymphoma.^{167–170} BCL2i are approved for relapsed or refractory chronic lymphocytic leukemia (CLL) with 17p deletion. They induced apoptosis in patient-derived CTCL cells *in vitro*, and this effect resulted synergistically potentiated using HDACi in combination.^{171,172} In addition, drug cocktails based on BETi and other epi- and/or non epi-targeted agents, such as the combination of the BETi JQ1 **42** and the BCL2i navitoclax during the treatment of MYCN-amplified SCLC,¹⁷³ or the combination of vorinostat **3** or romidepsin **4** and venetoclax against CTCL,¹⁷² have been reported for the treatment of solid and multiple hematologic tumors.

Kim *et al.* showed that the cytotoxic effects induced by BETi (such as JQ1 **42** or ABBV-075 **43**)¹⁷⁴ resulted synergistically amplified by either HDACi (vorinostat **3** or romidepsin **4**) or BCL2i (venetoclax **44**) (Combination J, Figure 3) in the majority of both CTCL cell lines (MyLa, Sez4, HH, Hut78) and CTCL tumor samples derived from patients who had previously tried both single and multiple therapies without success. In this study, synergistic effects on the modulation of the genes BCL2 and MYC, and of genes encoding for pro-apoptotic and cell cycle regulation factors (BIM, *CDKN1A* and *p21*), as well as a lower level of *BCL2L1* expression were observed.¹⁷⁵ In addition, a substantial increase in caspase 3/7 activation was registered when the BETi JQ1 **42** was combined with either romidepsin **4** or vorinostat **3**, confirming that the observed synergies are mainly due to apoptosis induction.¹⁷⁵

Preclinical studies conducted by Wang and coworkers¹⁷⁶ demonstrated the therapeutic potential of the combination of EZH2i and HDACi against small cell carcinoma of the ovary hypercalcemic type (SCCOHT), a rare but extremely lethal cancer that interests mainly young women. This kind of tumor shows a diploid genome with loss of SMARCA4 and lack of SMARCA2 expression, two mutually exclusive ATPases of the SWI/SNF chromatin-remodeling complex. Previously, preclinical studies showed that a subset of SCCOHT

patients took advantage from oncolytic virus¹⁷⁷ or c-Met inhibitors.¹⁷⁸ In agreement with the known antagonism between the PRC2 and the SWI/SNF complex, it has been recently demonstrated that SCCOHT cells are highly sensitive to EZH2 inhibitors, being EZH2 the catalytic subunit of the PRC2 complex.^{179,180} However, the EZH2i tazemetostat **19** only induced disease stabilization or partial response in two SCCOHT patients previously treated with a standard chemotherapy (www.epizyme.com). Such failure can be explained by the fact that SCCOHT cells survival depends on additional epigenetic pathways, whose identification and targeting could help in improving the response of SCCOHT cells to EZH2 inhibition. Through a rational epigenetic drug screen, Wang *et al.* highlighted that pan-HDACi markedly suppress SCCOHT cells growth. In fact, in this cancer type, pan-HDACi such as quisinostat **13**¹⁸¹ reversed the expression of a group of deregulated proteins, as a consequence of the SMARCA2 and SMARCA4 deficit, inducing apoptosis, differentiation and growth arrest *in vitro*, and suppressing growth of xenograft tumors of SCCOHT cells. A combined treatment based on the use of quisinostat **13** and tazemetostat **19** (Combination K, Figure 4) at sub-lethal doses synergistically induced targeted gene expression (such as genes dysregulated by SWI/SNF remodeling complex in SCCOHT) as a result of H3K27 hyperacetylation at the promoter region of PRC2, by simultaneous inhibition of both deacetylase and methyltransferase enzymes. Such H3K27 hyperacetylation led to growth suppression and apoptosis of both SCCOHT cells and xenografted tumors.¹⁷⁶

Recently, we reported a novel quinoline-based non-nucleoside DNMTi, MC3343 **45**, which showed promising preclinical effects against osteosarcoma. Our compound markedly induced cell proliferation arrest in both patient-derived cell lines and xenograft models. While the antiproliferative activity of MC3343 **45** was comparable with that of azacytidine **1**, its cell differentiating effect was much more evident. MC3343 **45** selectively increased the expression of osteoblastogenesis related-genes (including OCN, ALP and COL1A2) leading to matrix mineralization, an unregistered effect after azacytidine **1** treatment.¹⁸² In addition, MC3343 **45** has been tested in combination with two of the major chemotherapeutic drugs selected for osteosarcoma treatment, cisplatin and doxorubicin **36**, promoting doxorubicin-DNA bond as well as DNA damage and cell death. In particular, the doxorubicin **36**/MC3343 **45** combination (Combination L, Figure 4) inhibited PDX-OS#1-C4 cells growth by 65% after 24 h, compared to 32% growth inhibition obtained with doxorubicin **36** alone. Considering that none of these differentiating effects has been registered with azacytidine **1**, the use of MC3343 **45** alone and mostly in combination for osteosarcoma treatment could be very encouraging and should be further explored in the near future.¹⁸²

Soft tissue sarcomas (STSs) are rare forms of tumor involving different connective tissues originating primarily from mesoderm. Surgery with or without radiation therapy is the first choice for the treatment of localized sarcoma. Sarcoma growth depends on various factors, first of all neoangiogenesis,¹⁸³ therefore the application of specific antiangiogenic drugs, such as the recombinant human monoclonal antibody against vascular endothelial growth factor bevacizumab,^{184,185} has been studied in various clinical trials. In particular, the combination therapy based on the two cytotoxic drugs gemcitabine and docetaxel with bevacizumab was evaluated in patients with recurrent or advanced STS (). The treatment resulted safe, and the response rate was up to 31% for chemotherapy naïve patients and the

median response duration was about 6 months,¹⁸⁶ while treatment with bevacizumab alone resulted only in 13% response rate patients with epithelioid hemangioendothelioma or metastatic or advanced angiosarcoma.¹⁸⁷ Monga *et al.*¹⁸⁸ based on the idea that the pro-angiogenic effect of HDACs joined to tumor microenvironment could block the effect of bevacizumab, added VPA **8** to the combination of bevacizumab, gemcitabine **46**, and docetaxel **47** (Combination M, Figure 4), with the aim to increase the therapeutic response suppressing chemo resistance. Among the patients that did not respond to the treatment with gemcitabine/docetaxel alone, 61% responded (completely or partially) to the combination M. Furthermore, the addition of these two agents should be considered for therapeutic applications in patients whose disease progresses after two cycles of gemcitabine/docetaxel. This pilot study reveals the possibility to associate to the standard cytotoxic chemotherapy drugs that influence the tumor microenvironment, and pushes towards the repositioning of old agents. The most common adverse reactions that have been registered were hypertension linked to bevacizumab treatment, neuro and liver toxicity connected with VPA **8**, as well as cytopenia related to gemcitabine **46** and docetaxel **47** treatment. This combination appears to be moderately safe and of considerable potential in the next future.¹⁸⁸

Hypomethylating agents could increase the cytotoxicity of the anti-CD33 immunoconjugate gemtuzumab ozagamicin (GO)¹⁸⁹ against AML through the modulation of SHP-1 and Syk expression,^{190,191} as well as reducing the expression of p-glycoprotein which mediates the resistance to GO treatment.^{192–194} Based on these evidences, a phase I/II trial based on the combination of GO and azacytidine **1** (Combination N, Figure 4) was conducted ().¹⁹⁵ GO was approved in 2000 as monotherapy for the treatment of relapsed AML but was later retired from the US market (in 2011) for its lack of efficacy and toxicity. The aforementioned clinical study has been started before GO market withdrawal, and the dose (6 mg/m²) applied was the same used in therapy at that time. In 2017, GO was approved by the FDA in combination with citarabine **2** and daunorubicin for the treatment of AML, showing encouraging results. Fifty adult patients (29–82 years) affected by refractory or relapsed AML where initially treated with azacytidine **1** followed by GO. No dose-limiting toxicities were registered in phase I, however significant neutropenia and infection were the most common adverse reactions. While the optimal dose of GO remained unclear, the maximum tolerated dose adopted was of 75 mg/m² of azacytidine **1** (daily for six consecutive days) followed by 6 mg/m² of GO intravenously on days 7 and 21. After this treatment, 12 patients (24%) obtained complete remission or complete remission with incomplete peripheral blood recovery. These data are very similar to those obtained by administration of GO alone at higher doses, 9 mg/m² given twice.¹⁹⁶ Therefore, additional studies are required to determine the optimal schedule and dose of GO in combination with azacytidine **1**.¹⁹⁵

Histone methylation, as well as chromatin acetylation, are strongly implicated in androgen receptor (AR) protein functions and in mRNA splicing and transcription.^{197,198} In particular, the balance between HDACs and HATs activities is very important for tuning transcriptional regulation of AR and its downstream targets.^{199,200} HDACi modulate the expression of different factors and related pathways that play an essential role in cancer progression and resistance.^{197,198} Different evidences showed that VPA **8** increased caspase-2/3 activation

and decreased proliferation in AR-negative PC3 and DU145 cells.^{201–203} The most widely reported prostate cancer resistance mechanism is the upregulation of the AR.^{204–206} Preclinical shreds of evidence suggested that resistance to bicalutamide **48** treatment could be overcome through the simultaneous administration of panobinostat **6**, synergistically inducing apoptosis and growth arrest better than those obtained with panobinostat **6** alone, in both isogenic cell line and xenograft model of castration-resistant prostate cancer (CRPC).^{203,207–209} In 2018, a phase I/II clinical trial () conducted by Ferrari et al.²¹⁰ highlighted the capability of panobinostat **6** to restore sensitivity to bicalutamide **48** (Combination O, Figure 4) in a CRPC cancer model, as well as the safety and efficacy of its combination with bicalutamide **48** in CRPC second-line androgen resistant condition. The phase I trial was characterized by a 3 × 3 panobinostat **6** dose escalation design and the phase II trial involved randomized 55 patients treated with 20 or 40 mg of panobinostat **6** tri-weekly for 2 weeks in association with bicalutamide **48** 50 mg/day in 3-week cycle. The combination of 40 mg panobinostat **6** and bicalutamide **48** showed the best results inducing synergistic antitumor effect and reduced AR activity overcoming resistance as previously postulated.

AML development is highly frequent in elderly subjects, to whom the chemotherapy treatment, widely used for young patients, did not show the desired effects due to the common insurgence of pharmacological resistance²¹¹ and comorbidity with other diseases that preclude the correct functioning of different organs, compromising the whole condition.^{212–214} B-cell lymphoma 2 (BCL-2) protein overexpression has been found in leukemia stem cells,²¹⁵ and it has been associated with resistance to chemotherapy treatment and poor patients outcomes.²¹⁶ The potent and selective orally bioavailable BCL2 inhibitor (BCL2i) venetoclax **44** showed modest anticancer activity when evaluated as single therapy, since only 16 out of 32 patients affected by refractory and relapsed AML involved in the phase 2 clinical trial achieved an overall response.²¹⁷ Dose-escalation phase Ib study involving 57 elderly patients (> 65 years) based on venetoclax **44** and decitabine **2** or azacytidine **1** (Combination P, Figure 4) combination resulted well tolerated showing high survival and low early mortality ().²¹⁸ The dose-escalation stage has been conducted with the aim to evaluate the PK and the safety of this combination. More in detail, this phase involved three groups: group A (venetoclax **44** 400 mg (cohort 1) + intravenous decitabine **2** 20 mg/m²; days 1–5 of each 18-day cell); group B (venetoclax **44** 400 mg (cohorts 2 and 3) or 1200 mg (cohort 4) + intravenous or subcutaneous azacytidine **1** 75 mg/m²; days 1–7 of each 18-day cell); group C (venetoclax **44** 400 mg + decitabine **2** substudy with oral CYP3A inhibitor posaconazole **49** 300 mg twice on cycle – day 1 and 21 - and 300 mg once daily from cycle 1 – days 22–28). AML makes the affected subjects more susceptible to fungal infections, therefore posaconazole **49** resulted effective to prevent this condition.²¹⁹ Moreover, since venetoclax **44** is a substrate of CYP3A, group C was designed to evaluate the potential interaction of posaconazole **49** on the PK and safety of venetoclax **44** treatment. The collected evidences supported the use of the antifungal agent posaconazole **49** in combination with venetoclax **44** and decitabine **2** (Combination Q, Figure 4). Group A and B showed similar results in terms of safety and no dose-limiting toxicities, and the maximum tolerated doses were individuated but dose escalation was stopped at 1200 mg due to gastrointestinal toxicity. As a result, 60% of patients get complete remission or complete remission with incomplete marrow recovery at all dose levels in group A and B, and 65% in

the 400 and 800 mg dose cohorts with both selected hypomethylating drugs. The most common, dose-dependent adverse reaction recorded was the neutropenia due to venetoclax 44.²¹⁸

4. The MTDL approach.

Multi-targeting single drugs are the result of the conjugation of two or more active molecules that individually show a known activity against specific targets. This can be achieved in three different ways:^{3,4}

- connecting molecules with a linker that is cleaved in physiological conditions (mutual pro-drugs);
- connecting molecules with a stable linker, allowing each molecular moiety to interact with its specific target without interfering with the interactions established by the other portion;
- merging molecules together, preserving and connecting in the final MTDL only functional and reactive groups (warheads).

A MTDL could specifically act on one epi-target and another target not related to epigenetics, or on more epigenetic targets. Moreover, the active components of a MTDL can interact with molecular factors belonging to the same or different cellular pathways, in order to increase the therapeutic efficacy especially in diseases characterized by a frequent insurgence of acquired resistance during the therapy.

Most of the MTDL developed so far are hybrid molecules characterized by the scaffold of HDACi linked to another drug able to hit a related target involved in cancer. Pharmacophore portions of HDACi can be linked to other epi-drugs as well as to conventional cytotoxic agents (or to other drugs) to generate HDAC-based multi-target agents. The preference of HDACi's structural elements among various epi-drugs to design multi-targeting agents are due to the following reasons: i) in the 2000–2010 years there was a boom of studies on HDACi by medicinal chemists belonging to both industrial and academic institutions, and many HDACi characterized by a wide structural variety have been reported; ii) the cap group that characterizes all HDACi lays out of the catalytic tunnel of the enzymes and is tolerant of a high degree of structural variation without compromising the enzyme inhibition activity.^{220,221} This allows the introduction in this portion of a specific warhead able to interact with another target different from HDACs.^{3–5}

4.1 Dual HDAC/kinase inhibitors

Initially, in the tyrosine kinase inhibitors (TKi) drug discovery field, the aim was to synthesize highly selective inhibitors. Today, it is known that better results can be obtained with TKi able to simultaneously hit different kinase targets. In fact, on the one hand it is true that in some types of cancer the deregulation of the activity of a specific kinase seems to be the driving force. On the other hand, the treatment based on the use of a selective TKi may be useful, but after a prolonged treatment it can lose its efficacy because of new mutations and redundancies in biological networks that can compensate the drug effects via the activation of new aberrant cellular pathways. Thus, simultaneous inhibition of several

kinases has been recently considered as the best choice for a successful treatment.^{222,223} Furthermore, since co-administration of HDACi and TKi in *in vitro* and *in vivo* models have already shown synergistic effects (see above), the design of dual HDAC/TK inhibitors has become the most popular choice for the design of epigenetic multi-target drugs.^{224,225} Some of the approved TKi were a source of inspiration for the design of novel dual HDAC/TK inhibitors. These hybrid compounds are characterized by a hydroxamate or a benzamide moiety, that acts as HDAC inhibiting zinc binding group, connected to kinase inhibitors by different linkers.

Two HDAC/TK dual inhibitors entered clinical trials for cancer treatment. One of them, CUDC-101 **50** (Figure 5) was obtained by chemical manipulation of erlotinib **51** (Figure 5) through the introduction of the hydroxamate function separated from the quinazoline scaffold by a hexamethylene linker (typical of vorinostat **3**). CUDC-101 **50** inhibited HDACs as well as EGFR, platelet-derived growth factor receptor (PDGFR), and the human epidermal receptor 2 (HER2) in the nanomolar range.²²⁶ Furthermore, it regulated the proliferation and migration state of **51**-resistant NSCLC HCC827 cells through the down-regulation of E-cadherin, hepatocyte growth factor receptor (MET), protein kinase B (AKT) and human epidermal growth factor receptor 3 (HER3) compensatory pathways, which enable cancer cells to escape the effects of conventional EGFR/HER2 inhibitors.²²⁷ Moreover, CUDC-101 **50** induced caspase-dependent apoptosis in anaplastic thyroid cancer (ATC) leading to inhibition of cell proliferation and migration *in vitro*, through inhibition of MAPK signaling and histone deacetylation.²²⁸ CUDC-101 **50** is currently in phase I clinical trials to evaluate its safety and oral tolerability in cancer patients (), and in phase Ib open label study in patients with advanced head and neck (alone or in combination with radiotherapy and cisplatin,),²²⁹ liver, gastric, and breast cancer (), as well as NSCLC ().
228–230

The phosphatidylinositol 3-kinase (PI3K) plays an essential role in physiological functions such as proliferation, differentiation, protein synthesis, motility, and apoptosis.²³¹ When activated, it is also responsible for the onset of numerous diseases such as indolent BCL and aggressive BCL. Single-drug therapies with either PI3Ki or HDACi were often ineffective for the treatment of diffuse large BCL, because of concurrent activation of other survival- and growth-related pathways, while simultaneous inhibition of the two targets, PI3Ki and HDACi, displayed a synergistic effect. CUDC-907 **52** (Figure 5), the dual PI3K/HDAC inhibitor based on the structure of the PI3K inhibitor pictilisib **53** (Figure 5), is an orally active dual inhibitor potent at nanomolar level against both PI3Ks and HDACs [IC₅₀ values = 19 (PI3K α), 54 (PI3K β), 1.7 (HDAC1), 5 (HDAC2), 1.8 (HDAC3), 2.8 (HDAC10) nM]. CUDC-907 **52** was effective against chronic lymphocytic leukemia (CLL) through PI3K/HDAC inhibition joined to suppression of STAT3 and RAF/MEK/ERK signaling, thus reducing the expression of anti-apoptotic BCL2-family members MCL-1, BCL-XL and BCL-2. In addition, cytokines such as APRIL and BAFF as well as their receptors BMCA, TACI and BAFFR resulted downregulated after the treatment, blocking, by this way, NF- κ B and BAFF-induced signaling. Thus, the pleiotropic effects induced by CUDC-907 **52** abrogate different protective and pro-survival signals, rescinding microenvironment protection and highlighting the possibility of sensitizing CLL cells to other drugs.

Accordingly, a low concentration combination therapy based on CUDC-907 **52** and different inhibitors of NF- κ B (IMD-0354), BTK (ibrutinib) and BCL2 (ABT-199) signaling pathways leads to a potent synergistic effect.²³² Different pre-clinical data in hematologic and solid cancer cell lines have shown that the effects obtained with CUDC-907 **52** in terms of tumor growth inhibition and multiple pro apoptotic activities are more potent than those achieved using single PI3K or HDAC inhibitors. In particular, a treatment with CUDC-907 **52** gave a reduction in MYC gene expression in MYC-driven tumor model, whose overexpression has been reported to be among the worst prognostic factors in relapsed refractory diffuse large B-cell lymphoma (DLBCL).²³³ Dose escalation and expansion of CUDC-907 **52** in DLBCL and MYC-altered disease, with and without the monoclonal antibody rituximab (25 patients received monotherapy treatment while 12 received the combination) () showed a median duration of the overall response of 11.2 months (of 13.6 months in MYC-altered patients, 7.8 months in those with unknown MYC status, and 6.0 months in MYC unaltered patient), a durable antitumor activity (in particular in MYC-altered patients) as well as a safety and tolerable profile.²³⁴ CUDC-907 **52** has been reported to downregulate FLT3 expression in AML cells. Recent preclinical studies conducted by Knight *et al.*²³⁵ confirmed that **52** synergistically increased the apoptosis induced by the novel dual FMS-like tyrosine kinase 3 (FLT3) and AXL receptor tyrosine kinase (AXL) inhibitor gilteritinib in both FLT3-ITD AML cell lines and FLT3-ITD patient samples. Such synergistic antileukemic activity occurred thanks to the downregulation of gilteritinib-induced FLT3 expression in AML cells mediated by CUDC-907 **52**. In addition, this combination cooperatively inhibits the JAK-STAT, PI3K-AKT and RAS-RAF signaling pathways, preventing the escape via alternative pathways and providing a strong foundation for subsequent *in vivo* murine studies. To date, CUDC-907 **52** is in phase I clinical trials for the treatment of solid tumors and lymphomas (, , ,).²³⁶

Activation of phosphatidylinositol-3-kinase-Akt-mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway is responsible for the onset of hepatocellular carcinoma (HCC), and of the acquired resistance to sorafenib, the first and the only approved systemic TKI drug^{237,238} for the treatment of this type of aggressive cancer. In April 2017 a fluorinated analog of sorafenib, known as regorafenib has been approved for the treatment of sorafenib-resistant HCC patients,^{239,240} but no drugs able to overcome both types of drug-resistance are known today.²⁴¹

In literature it has been reported that the upregulation of class I HDACs is a common factor in HCC contributing to spread, aggressiveness and increased mortality.^{238–241} Recently, Cheng *et al.*²⁴² reported a series of purine-based hydroxamates able to interact with both HDAC and PI3K/Akt/mTOR signaling pathway for the treatment of HCC. As highlighted through previous structure-activity relationship (SAR) studies focused on the PI3K/mTOR inhibitor **54** (Figure 6),²⁴³ the introduction of a reasonably sized substituent, specifically of a hydroxamate portion at the N9-position, resulted well tolerated reaching the desired dual inhibitory capability. Among the several analogues obtained through different manipulations at the positions C2, C6, N7, and N9 of the bicyclic system, the most potent compound **55** (Figure 6) showed lower IC₅₀ values against both HDAC1 (IC₅₀ = 1.1 nM) and PI3K δ (IC₅₀ = 37 nM) than the relative single targeting compounds [vorinostat **3**: IC₅₀ = 12 nM

(HDAC1), **54**: IC₅₀ = 340 nM (PI3Kβ)]. Accordingly, **55** displayed higher or similar antiproliferative activity in MV4–11 leukemia cell lines [IC₅₀ = 0.047 μM vs 1.46 μM (pictilisib **53**) and 0.043 μM (vorinostat **3**)] and in the hepatic carcinoma cell lines HuH-7 [IC₅₀ = 0.48 μM vs 1.72 μM (pictilisib **53**) and 1.96 μM (vorinostat **3**)]. Compound **55** showed excellent oral anti-tumor activity as a single agent in HCC and 4T1 mouse metastatic breast cancer models and these results, together with the good PK characteristics, suggested the potential use of **55** either as monotherapy or, preferably, in combination with well-known approved (immuno)drugs for treatment of non-Hodgkin lymphomas (i.e., diffuse large B-cell lymphoma) and subgroups of leukemia (i.e., AML). Furthermore, compound **55** proved to be safe as it did not inhibit hERG channel protein (IC₅₀ >100 μM), and no significant inhibition of CPY3A4 (IC₅₀ >10 μM) has been registered.²⁴²

Fibroblast growth factor (FGF) family and their four receptor tyrosine kinases (FGFR1,2,3,4) cover an essential role in many physiologic processes such as tissue homeostasis and repair, embryogenesis, inflammation, and wound healing. However, the amplification of their expression was found in different kind of cancers such as breast, gastric, cervical, oral or bladder cancer.^{244–252} Recently, the combination composed by HDAC6 and TK inhibitors was proven to be effective against breast cancer, suggesting the possible development of hybrid compounds able to interact with both targets.^{253,254} Compound **56** (IC₅₀ FGFR1 = 2.9 nM) (Figure 6) has been previously reported by Liu *et al.*²⁵⁵ as novel and selective second generation TKi, and resulted the starting point for the design of the first series of HDAC6/FGFR1 dual inhibitors.²⁵⁶ The **56**/FGFR1 co-crystal structure revealed that the *N*-ethyl-4-phenylpiperazine moiety extended out of the solvent exposed region. Thus, the *N*-ethyl-4-phenylpiperazine portion was replaced with the Zn²⁺ binding group of the HDAC6 selective inhibitor nexturastat A **57** (IC₅₀ HDAC6 = 6 nM, with >190-fold selectivity over other HDACs) (Figure 6),²⁵⁷ obtaining several potential hybrid molecules among which the most potent compound was **58** (IC₅₀ HDAC6 = 34 nM, 64% inhibition of FGFR1 at 1 μM) (Figure 6). Antiproliferative data in breast cancer MCF-7 cells collected with **58** (IC₅₀ value = 9 μM) were comparable with those of vorinostat **3** (IC₅₀ = 2.7 μM) and nexturastat A **57** (IC₅₀ = 1.4 μM).

Cyclin-dependent kinases (CDKs) play a major role in modulating cell cycle regulation. In particular, CDK4 controls the G₁ phase through the formation of a complex with cyclin D1. Overexpression of CDKs and in particular of CDK4, as well as dysregulation of the CDK4-cyclinD-Rb pathway, have been registered in cancer. CDK4 inhibitors (CDK4i) could induce arrest in G₁ phase and in cell cycle progression controlling tumor growth.^{258–264} The first generation of CDK4i showed poor specificity and severe toxic side effects that limited their use in clinical trials.^{265,266} Therefore, highly selective CDK4i were developed such as ribociclib and palbociclib, which entered clinical trials.^{267–271} The combination of CDKi with the aromatase inhibitor letrozole resulted essential for the treatment of ER⁺/HER2[–] metastatic breast cancer, suggesting that their use as single agents may be insufficient.^{272,273} The synergistic effect resulting from the combination of HDACi and CDKi in melanoma and neuroblastoma²⁷⁴ induced Li *et al.*²⁷⁵ to develop hybrid molecules based on the pyrrolo[2,3-*d*]pyrimidin-2-ylamine scaffold of the CDK4/9 inhibitor ribociclib **59** (IC₅₀ CDK4 = 13 nM). By the introduction of the anilide-polymethylene-hydroxamate motif (typical of

vorinostat **3**) at the C2-amino group of ribociclib **59**, a series of novel, highly selective (in a kinase profiling assay against 375 kinases) and potent inhibitors of both CDK4/9 and HDAC1 has been obtained. Compound **60** (Figure 6) was the most effective of the series, with IC₅₀ values of 8.8 (CDK4), 12 (CDK9), and 2.2 (HDAC1) nM. In cells, **60** induced cytotoxic effects and apoptosis in different cancer cell lines (liver Hep3B and HepG2, breast MDA-MB-231, 4T1, and T47D, and lung H460 and A549 cancer cell lines) through CDK4/9 and HDAC1 inhibition and phosphorylation of p53 in addition to G₀/G₁ (at low concentration) or G₂/M (at high concentration) arrest, thus preventing cell differentiation and proliferation. Oral or intraperitoneal administration of **60** led to a potent tumor regression in mice bared-breast cancer 4T1 xenograft models. Additional studies showed that **60** downregulated the activity of CDK4 as well as the phosphorylation of protein Rb and significantly upregulated the acetylation state of H3 and the relative levels of antiapoptotic BCL-2 expression and cleaved-caspase-3.²⁷⁵

Huang and coworkers demonstrated that the activity of vorinostat **3** resulted synergistic with that of the CDK4/8 inhibitor abemaciclib **61** (Figure 6) against different solid tumors.^{276,277} X-ray co-crystal structure analysis of abemaciclib **61** and CDK4/CDK6 revealed that the 2-aminopyrimidine group which interacts with the protein hinge region was important for the **61** inhibitory activity. Therefore, a series of dual CDK/HDAC inhibitors was developed merging the pharmacophoric scaffold of abemaciclib **61** with that of vorinostat **3** or similar.²⁷⁸ Among the synthesized compounds, Roxyl-zhc-84 **62** (Figure 6) showed the most potent antitumor activity against a broad spectrum of solid tumors in both *in vivo* and *in vitro* models, showing improved HDAC isoform selectivity profile compared to the pan-HDACi vorinostat **3**. In cells, Roxyl-zhc-84 **62** showed high cytotoxic activity (IC₅₀ values in the 10–40 nM range in breast 4T1, MDA-MB-468 and MDA-MB-231 and ovarian SK-OV-3, OVCAR-5 and H450 cancer cell lines, 30 times lower respect to vorinostat **3**. In addition, **62** was well-tolerated in *in vivo* assays (in both 4T1 and MDA-MB-468 mouse models), with no significant loss of body weight. At molecular level, Roxyl-zhc-84 **62** reduced STAT3 and Janus kinase 1 (JAK1) phosphorylation markedly downregulating BCL-2, which is known to be a STAT3 target gene, thus sensitizing cells to HDACi activity. In addition, due to its HDAC inhibitory capability Roxyl-zhc-84 **62** increased the expression level of the cell cycle regulator p21. Importantly, Roxyl-zhc-84 **62** showed a stronger antitumor activity than the combination of abemaciclib **61** and vorinostat **3**, or than that of JAK1i (filgotinib) and HDACi in both *in vitro* and *in vivo* models.²⁷⁸

The vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor (VEGFR) signaling pathway is one of the most important targets for anti-angiogenesis and counts over ten small molecule inhibitors and monoclonal antibodies adopted for the treatment of solid tumors.^{279–281} Pazopanib **63** (Figure 6) is a multiple VEGFR inhibitor approved by the FDA for the treatment of advanced soft tissue sarcoma and advanced renal cell carcinoma.²⁸² Unfortunately, the clinical application of pazopanib **63** is limited by the insurgence of resistance and tumor relapse.²⁸³ Several phase I clinical studies supported the combination therapy focused on the co-administration of pazopanib **63** and different HDACi such as vorinostat **3** or abexinostat **11**.²⁸⁴ Hence, based on these evidences, Zang *et al.* designed and synthesized a series of dual VEGFR/HDAC inhibitors by replacing the **63**

benzene sulfonamide moiety with either a *ortho*-aminoanilide or hydroxamate group.²⁸⁵ The most potent analogs **64** and **65** (Figure 6) were evaluated for their antiproliferative activity against four hematological and seven solid tumor cell lines. In this assay, they showed potent arrest of cell proliferation while pazopanib **63** was not effective, highlighting the need for dual inhibition to obtain the desired effect with these compounds. In HUVECs tube formation assay and rat thoracic aorta rings assay, **64** and **65** strongly inhibited the microvessels outgrowth showing comparable potencies to pazopanib **63**. Among the two hybrid compounds, only **64** progressed to *in vivo* PK studies showing 72% oral bioavailability in rats, while **65** suffered from metabolic liability due to the hydroxamate function. When tested in human colorectal adenocarcinoma (HT-29) xenograft model, **64** displayed considerable *in vivo* antitumor efficacies with no significant toxicity or body weight loss.²⁸⁶

Yao *et al.*²⁸⁷ developed a triple inhibitor able to hit distinct targets showing related functions in cancer development: JAK2 kinase, heat-shock protein 90 (HSP90), and HDACs. HSP90 is a molecular chaperone that assists a set of proteins (involved in signal transduction such as kinases and steroid hormone receptors as well as oncoproteins) during their maturation ensuring the correct folding.¹⁴² In addition, HSP90 resulted strongly upregulated in cancer inducing the activation of STAT3, an oncogenic inflammatory mediator.^{288,289} Since also JAK kinase activate STATs,²⁸⁸ and HDACs (in particular HDAC6) play an important role in modulating the HSP90 complex,²⁹⁰ the development of a triple JAK/HSP90/HDAC inhibitor able to interact simultaneously with all the three targets has a strong rationale. It is known that in the structure of the JAK inhibitor ruxolitinib **66** (Figure 6)^{291,292} (IC₅₀ values = 3.3 (JAK1) and 2.8 (JAK2) nM) the C2 position of the 7-deazapurine, as well as the substituent at the C6 pyrazole group, can be modified without losing the inhibitory activity. Hence, these positions have been functionalized introducing the pharmacophoric elements typical of the HSP90 inhibitor BEP800 **67** (IC₅₀ HSP90 β = 58 nM), that does not suffer modifications on the *meta* position of the phenyl ring for its inhibiting activity (Figure 6),^{293,294} and vorinostat **3**, for which the possibility to functionalize the phenyl C4-position is well known.

Among the synthesized derivatives, **68** (Figure 6) showed the highest potency, inhibiting all the desired targets in the low micromolar range (IC₅₀ values: 3.76 (JAK2), 20.2 (HSP90), and 6.3 (HDAC6) μ M). Since this is a very recent and early state project, further chemical optimizations and biological analyses are required.²⁸⁷

4.2 Dual LSD1/kinase inhibitors

Osimertinib **69** (Figure 7) is an orally administrated, FDA-approved irreversible third generation EGFR inhibitor for the treatment of EGFR-mutated NSCLC. Recently, Li *et al.*²⁹⁵ reported that this well-known TKi is a dual EGFR/LSD1 inhibitor, displaying a IC₅₀ against the KDM LSD1 of 3.98 μ M due to its capability to act as a FAD competitor or FAD ejector (although not structurally similar to FAD). In NCI-H1975 cells, osimertinib **69** enhanced in a dose-dependent way the H3K4me1/2 levels as well as the expression of CD86, known to be biomarkers to evaluate LSD1 inhibition in cells, and arrested cells migration confirming its dual inhibition capability. Considering the important role that LSD1

covers during the onset of NSCLC, osimertinib **69** could be a new promising strategy for NSCLC treatment.

4.3 Dual BET/kinase inhibitors

Several kinase subtypes, as well as some members of the BET family, are implicated in the same pathways that characterize specific cancer pathologies. For example, FMS-related tyrosine kinase 3 (FLT3) and BRD4 are key targets in AML,²⁹⁶ and both BRD4 and JAK kinases play an important role in multiple myeloma models.^{168,297} The co-administration of a known TKi such as the FLT3 inhibitor quizartinib and JQ1 exhibited synergistic effects in cancer cell lines expressing the FLT3-ITD mutation (AML cells and AML stem/blast progenitor cells).^{298,299} SB284851-BT **70** (Figure 8) belongs to the 1,4,5-trisubstituted imidazoles mainly known as p38 α kinase inhibitors. For these compounds, the ability to bind also BET bromodomain has been reported (IC₅₀ values = 18 [BRDT (domain 1)], 3.7 [BRD4 (domain 1)], >250 [BRDT (domain 2)] μ M, and not determined [BRD4 (domain 2)]).^{300–302} Starting from SAR studies focused on **70** (Figure 8), in 2018 Divakaran and coworkers³⁰³ identified a new series of selective BRD4 (domain 1) over BRD4 (domain 2) (>55 fold) 1,4,5-trisubstituted imidazoles as dual p38 α -bromodomain inhibitors. The properly substituted imidazole scaffold binds to bromodomain proteins in a similar manner respect to a histone acetyl group, taking part in a hydrogen bond interaction with conserved asparagine residues (N140) through its nitrogen atom. The selectivity is due to the flexibility of Lys141 typical of BRD4 (domain 1), compared to the steric limitation of the histidine typical of the C-terminal BET bromodomain and BRD4 (domain 2), that allows the displacement of structured water molecules (carried out by the fluorophenyl ring able to penetrate deeper into the binding pocket respect to other BETi), in addition to the effects induced by the imidazole ring itself. The obtained compounds, in particular **71** (Figure 8), showed, in addition to a potent and selective inhibition for domain 1 of BRDT, BRD4, BRD2, and BRD3 as well as for p38 α (IC₅₀ values = 3.5 [BRDT (domain 1)], 1.7 [BRD4 (domain 1)], 29 [BRD2 (domain 1)], 11 [BRD3 (domain 1)], >250 [BRDT (domain 2)], >250 [BRD4 (domain 2)], 67 [BRD2 (domain 2)], >100 [BRD3 (domain 2)] μ M; k_d p38 α = 0.47 nM), the capability to efficiently bind BRD4 in different cell lines, and an excellent cell permeability. The A459 lung cancer cell line showed improved sensibility to the treatment, due to the capability of these molecules to target both p38 α and BRD4 (demonstrated by decreased cytokine secretion). In contrast, the effect on the multiple myeloma MM.1S cell line resulted largely BRD4-driven, due to the prevalent sensibility of this cell line to the modulation of c-Myc expression, a characteristic hallmark of BETi. Early evidences also showed that such cooperative effects are the consequence not only of the dual inhibitory activity of these molecules, but also of the selectivity of their action among the domain 1 of BET proteins.³⁰³

4.4 Hybrids compounds able to simultaneously inhibit epigenetic and not epigenetic/not kinase targets

Some dual compounds have been designed by coupling the HDACi pharmacophore group with structures of inhibitors of various enzymes, apart from kinases. Two main factors have been identified at the base of the onset of acute promyelocytic leukemia (APL): the fusion of the RAR α protein with the promyelocytic leukemia gene (PML), and the presence of blasts

blocked at the promyelocytic stage. The effectiveness of the treatment based on ATRA is due to the capability of this drug to release the HDAC-containing repressive complex bound to PML-RAR α with the recruitment of the multi-subunit HAT complex on specific DNA responsive elements (RARE). Based on the evidence that ATRA induces differentiation and cell death in APL cell lines, and assuming the remarkable context-specific physical proximity of a retinoid and HDACi-binding protein in the repressive PML-RAR α -HDAC complex, we pioneered the epi-polypharmacology field with design and synthesis of the hydroxamate **72**³⁰⁴ (Figure 9) and the benzamide **73**³⁰⁵ (MC2392, Figure 9), strictly related to ATRA, to be tested in leukemia. While **72** did not show any significant activity in leukemia cells, **73**, despite its weak retinoid activity and general no HDAC inhibition *in vitro* and *in vivo*, was able to induce changes in H3 acetylation at a small subset of PML-RAR α -binding sites, to alter expression of stress-responsive and apoptotic genes, and to induce massive cell death specifically in PML-RAR α expressing APL cells, while solid and other leukemic tumors were not affected.³⁰⁵

The activation of T cells in order to obtain a native immune response through immune check point therapy is a very interesting strategy that has achieved significant advances in clinical trials for cancer therapy.³⁰⁶ Indoleamine-2,3-dioxygenase (IDO1) is an extrahepatic heme-containing dioxygenase able to catalyze the conversion of tryptophan (Trp) to *N*-formylkynurenine (NFK), that is then converted to L-kynurenine (Kyn) and different bioactive metabolites.³⁰⁷ The reaction in which IDO1 is involved is the rate-limiting step of the kynurenine pathway (KP).^{307,308} T lymphocytes proliferation is dependent on the Trp levels, so Trp depletion induces its inhibition. In addition, the metabolites deriving from KP can activate the aryl hydrocarbon receptor (AhR) inducing immune tolerance. Both of these conditions contribute to the immunosuppressed state at the base of tumor pathogenesis,^{309,310} and high levels of IDO1 (in both antigen presenting and tumor cells) are often correlated with reduced survival.^{311,312} IDO1 inhibitors (such as epacadostat, IC₅₀ = 10 nM) eradicate and control cancer cells growth due to their capability to induce antitumor immune response, but a lot of preclinical studies showed that their activity is reduced when these molecules are used as single agents.^{311,313} Conversely, IDO1 inhibitors improved their efficacy when administered in combination with radiopharmaceutical drugs, cytotoxic anticancer agents, etc.^{311,313,314} Recent evidences showed that HDACi could be able to reverse immune suppression inducing an improvement in tumor recognition.^{315,316} Fang *et al.* reported the first dual HDAC/IDO1 inhibitors, designed by insertion of the benzamide anti-HDAC moiety of entinostat **9** or mocetinostat **10** into the structure of epacadostat **74**, through replacement in its molecule of the aminoethyl-sulfamide function, exposed to the solvent in the binding with the enzyme.^{317,318} The dual agent **75** (Figure 9) inhibited both HDACs and IDO1 at nanomolar range, decreased proliferation, and induced G₂/M cell cycle arrest and apoptosis in HCT-116 colon, LLC lewis lung, and A549 lung cancer cell lines. Moreover, **75** showed an acceptable PK profile after oral administration reducing plasma Kyn levels over 8 h with an interesting anticancer activity and low toxicity *in vivo* in a murine LLC tumor model.³¹⁸

Considering the high request of NAD⁺ by cancer cells, the inhibition of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in NAD⁺ biosynthesis, has

been considered a potential strategy to hit cancer.^{319,320} The NAMPT inhibitor (NAMPTi) FK866, which entered clinical trials but whose development has been limited by dose-limiting thrombocytopenia,^{321–324} has been reported to synergistically improve the activity of HDACi. Starting from these evidences, Chen *et al.*³²⁵ developed dual NAMPT/HDAC inhibitors, by connecting the *N*-(3-(1*H*-imidazol-1-yl)propyl)-5-thiazolecarboxamide moiety contained in the novel NAMPT inhibitor **76** (Figure 9)³²⁶ with an amidopolymethylene-hydroxamate tail, able to inhibit HDACs.³²⁷ Among them, **77** was the most potent compound in term of enzymatic and cellular activity (IC₅₀ values: 15 (NAMPT), 2 (HDAC1), 6.8 (HDAC2), 0.67 (HDAC3), 12000 (HDAC4), 0.64 (HDAC6) and 204 (HDAC8) nM) (Figure 9). Specifically, **77** was slightly less potent than the NAMPTi FK866 and strongly inhibited better than vorinostat **3** all the tested isoforms of HDACs except HDAC4. Accordingly, **77** markedly increased the acetylation levels of histones H3 and H4 in a dose-dependent manner, and efficiently decreased the cellular level of NAD⁺ after 24 h of incubation in human colon cancer cells HCT116. In cells, **77** induced moderate cytotoxicity against HCT116, HepG2 liver cancer, and A549 lung cancer cell lines. However, when evaluated *in vivo* in HCT116 tumor xenografts in nude mice through intraperitoneal infusion at 25 mg/kg twice a day for 21 consecutive days, **77** determined a strong reduction of tumor growth, and higher or comparable antineoplastic activity (TGI = 42%, T/C = 58%) respect to FK866 (TGI = 39%, T/C = 61%) and vorinostat **3** (TGI = 33%, T/C = 67%), without significant body weight loss.³²⁵

Recent evidences highlighted the capability of fluoroquinolones to carry effective broad-spectrum antitumor activity against different types of cancer such as SLCL, colorectal carcinoma, and bladder cancer.^{328,329} In particular, levofloxacin **78** (Figure 9) showed antiproliferative activity against a different kinds of tumors.³³⁰ In addition, many fluoroquinolones inhibited tubulin polymerization exhibiting selective activity against some tumor cell types^{330,331} and favorable PK characteristics.³³²

Wang *et al.*³³³ developed a series of hybrid HDAC/tubulin polymerization inhibitors based on the levofloxacin **78** scaffold. Across this series, **79** (Figure 9) was more potent than vorinostat **3** against HDAC1 and HDAC6, and more effective than levofloxacin **78** and colchicine in inhibition of tubulin polymerization (ITP). Furthermore, **79** exhibited higher antitumor activity and cell type selectivity respect to the corresponding single target inhibitors when tested against different kinds of tumor cell lines, and in particular against MCF-7 breast cancer cells (IC₅₀ **79** = 0.3 μM, IC₅₀ vorinostat **3** = 4.4 μM, IC₅₀ levofloxacin **78** = 64.2 μM).

Recently, the combination of vorinostat **3** plus vincristine has been shown to induce synergistic effects in both *in vitro* and *in vivo* models, suggesting that vorinostat **3** could alter microtubule dynamics through HDAC inhibition.³³⁴ Thus, another series of dual HDAC/tubulin polymerization inhibitors has been successfully developed by Lamaa and coworkers by merging the structure of belinostat **5** with the 1,1-diarylethylene scaffold of *iso*-combretastatin A-4 **80** (IC₅₀ for ITP = 2.0 μM) (Figure 9).³³⁵ Among these compounds, **81** (Figure 9) showed the best antiproliferative results (IC₅₀ = 0.4–2 nM) in different tumor cell lines (K562, PC3, U87, CA-4, HT-29, BXP3), inducing a strong inhibition of both

HDAC8 ($IC_{50} = 340$ nM) and tubulin polymerization ($IC_{50} = 1.6$ μ M) as well as cancer cells cycle arrest at the G₂/M phase by disruption of the microtubule organization.³³⁵

As previously reported, recent evidences highlighted the synergism deriving from the combination of HDAC6-selective and HSP90 inhibitors. The 4-isopropylresorcinol scaffold is known to fit into the hydrophobic and hydrophilic region of HSP90 playing a key role as binder at the ATP binding site.^{336–338} Merging such evidence with those that indole and non-planar indoline/tetrahydroquinoline-based scaffold act as a selective HDAC isoform inhibitor, a series of 1-aryloindoline-hydroxamic acids differing in the length of the linker have been developed by Oiha *et al.* as dual HDAC/HSP90 inhibitors.³³⁹ Among these compounds, **82** (six methylene linker) (Figure 9) was the most potent with IC_{50} values of 46.3 nM for HSP90 and 1.15 nM for HDAC6 (with a 113, 139 and 246-fold selectivity over HDAC1, HDAC3 and HDAC8 isoforms, respectively). In colorectal HCT116, lung A549, EGFR T90M mutant lung H1975 and leukemia HL60 cell lines, **82** displayed cytotoxic effects with $GI_{50} = 1.04$ – 1.61 μ M, and the maximum cytotoxic effect was registered towards H1975 NSCLC cell lines with $IC_{50} = 0.26$ μ M.

As reported in literature, HDAC6 and class I HDACs cover an essential role in Alzheimer disease (AD), since their overexpression were detected in the cortex and hippocampus of AD patients.^{26–30} It has been postulated that at the basis of the onset and progression of AD there is the aberrant aggregation of the insoluble hyper-phosphorylated Tau protein (pTau). In particular, the acetylation of the KXGS motif of this protein (at the level of Lys280) prevents tau aggregation as a result of the inhibition of the phosphorylation process. Recent evidences showed that HDAC6 is responsible for deacetylation of these residues, therefore blood brain barrier (BBB)-permeable HDAC6 inhibitors could be very useful for the enhancement of this acetylation so preventing tau aggregation.^{31–33} Also the oxidative stress contributed to the onset of different neurodegenerative disorders including AD.³⁴ HDAC6 plays a central role also in redox regulation and in cellular stress response: in fact, the overexpression of HDAC6 is responsible for ROS generation in response to the upregulation of the expression of NADPH oxidase as well as pro-inflammatory cytokines and α -tubulin deacetylation.³⁵ Selenium is a mineral nutrient physiologically present in essential traces in our organism, providing protection from free radicals. Its levels decrease during aging, covering different possible roles in AD.^{36,37} Starting from these evidences, Hu *et al.*³⁸ developed a series of compounds based on the structures of vorinostat **3** and of the antioxidant and anti-inflammatory agent ebselen **83** (Figure 9).³⁹ Among the synthesized compounds, **84** (Figure 9) showed the highest anti-HDAC6 activity ($IC_{50} = 0.037$ μ M), joined to comparable or better glutathione peroxidase-like activity than ebselen **83**, providing high free oxygen radical scavenging capability (ORAC = 2.2) in addition to marked protective activity against ROS accumulation in Rat adrenal pheochromocytoma PC12 cells.

The inhibition of phosphodiesterase 5 (PDE5) leads to an enrichment in the available concentration of the second messenger cyclic guanosine monophosphate (cGMP). It resulted critical for neuronal signaling, promoting memory-related gene transcription through the activation of the cAMP response element (CREB) and the consequent recruitment of CREB binding proteins (showing HAT activity) due to the increased phosphorylation state. Several

studies reported a level of cerebrospinal fluid cGMP lower in patients affected by AD than in age-matched controls.³⁴⁰ Clinical evidences of enhanced synaptic plasticity and memory improvement following PDE5 inhibitors administration (in particular, by using compounds able to cross the BBB such as sildenafil, tadalafil and vardenafil) in AD patients were collected during the last years.^{341–343} Combinations of tadalafil (IC_{50} PDE5A = 9.4 nM), sildenafil (IC_{50} PDE5A = 8.5 nM) (Figure 9)³⁴⁴ or vardenafil (IC_{50} PDE5A = 0.89 nM),³⁴⁵ and vorinostat **3** were pre-clinically validated in a mouse model of AD,³⁴⁶ showing a synergistic effect for increased levels of histone acetylation via recruitment of CBP, similarly to what observed by the treatment with previously discovered hybrid PDE5/pan-HDAC inhibitors in mice.³⁴⁷ The effect of the HDAC6-selective inhibitor tubastatin A in combination with PDE5Ai has been evaluated against SH-SY5Y neuroblastoma cell lines, with the aim to highlight the role of this specific HDAC isoform in such disease. A marked increase of H3K9 acetylation has been registered compared to cells treated with tubastatin A alone.³⁴⁸ From these findings, Rabal *et al.*³⁴⁸ recently identified new sildenafil **85**-based dual PDE5A/HDAC6 inhibitors. Among them, **86** (Figure 9) displayed the best enzyme inhibitory activity (IC_{50} HDAC6 = 15 nM; IC_{50} PDE5A = 11 nM), was able to cross the BBB and to achieve its functional response, reducing the levels of AD-related markers such as pTau and human amyloid precursor protein in Tg2576 neurons, and increasing the markers of the physiological neuron response in mouse hippocampus following the administration of 40 mg/kg dose. However, this treatment did not induce a significant improvement in memory after 2 weeks.³⁴⁸

Both HDACs and transglutaminases (TGs) play a pivotal role in neurodegeneration, and several evidences showed their induction in the presence of toxic stimuli. Moreover, the inhibition of both targets induces a protective effect in different neurodegenerative diseases restoring the mRNA and protein levels of brain-derived neurotrophic factor (BDNF), a well-known neurotrophin implicated in neuronal vitality, development and synaptic plasticity.³⁴⁹ In particular, the most expressed and studied TG isoform is TG2, which is mostly involved in AD and Huntington's disease (HD). A drug combination based on the TG2 inhibitor (TGi) B001 and the HDACi sodium butyrate, demonstrated that this co-treatment synergistically protects cells from glutamate toxic effects at the tested concentration (% viable cells: 28 (glutamate alone), 37.5 (glutamate plus B001), 44.3 (glutamate plus sodium butyrate), 67.5 (glutamate plus B001 and sodium butyrate)). On the same line, the combination based on cysteamine and vorinostat **3** markedly enhanced cell survival (from 28% for cysteamine alone and 36.7% for vorinostat alone to 59.8% for combination). To date, only a few TG2i have been reported in literature and, in particular, some 3-(substituted cinnamoyl)pyridines including compound **87** (IC_{50} TG2 = 21 μ M) (Figure 9) are worthy of note. In the **87** structure, the nitro group at the *para* position of the cinnamoyl moiety could be replaced by more complex substituents without loss of enzyme inhibitory activity. For the first time, Basso *et al.*³⁵⁰ developed a series of dual TG2/HDAC inhibitors based on the central scaffold of **87**. To support previously collected data about the synergistic effect induced by co-treatment of TG2i and HDACi, the best molecule among those synthesized, compound **88** (IC_{50} TG2 = 13.3 μ M, IC_{50} HDAC1 = 3.38 μ M, IC_{50} HDAC6 = 4.10 μ M) (Figure 9) was evaluated in cortical neurons to test its capability to protect neurons from death. Cells were treated with increasing concentration of **88** without further toxic stimuli.

Compound **88** did not show any toxic effects at the highest tested concentration (50 μ M), showing a neuroprotective effect at concentration as low as 6.3 μ M, where 80% of the neurons were preserved, and at higher concentrations a complete protective effect was achieved (at 25 μ M: 95% viable cells, at 50 μ M: 100% viable cells).³⁵⁰

Dystrophic mice treated with either NO donors or selective HDACi showed partial rescue of the disease, suggesting to develop dual NO donors/HDACi agents. This goal was reached by introducing the dinitrate NO donor moiety at the C6-position of the pyridine ring of entinostat **9**. Compound **89** (Figure 9) was the most potent among the series of hybrid compounds synthesized by Atlante *et al.*,³⁵¹ showing inhibitory values against HDACs comparable with those of entinostat **9**. In particular, the highest inhibitory activity of **89** was towards HDAC2 (IC₅₀ = 0.39 μ M), that is inhibited also by *S*-nitrosylation.³⁵² Compound **89** furnished also the best results in terms of NO donor activity according to cytometry and fluorescence-activated cell sorting (FACS) analysis, skeletal muscle differentiation, *ex vivo* (from male Wistar rats) aortic vasodilation, and formation of large myotubes characterized by a high number of multinucleated fibers. Compound **89** has been tested for its cytotoxic effect in immortalized mouse myoblast C2C12 cells and immortalized human keratinocyte HaCaT cells, showing lower cytotoxicity than entinostat **9**.³⁵¹

4.5. PROteolysis Targeting Chimera (PROTAC) approach applied to epigenetic targets

Thalidomide **90** (Figure 10) was originally used as a sedative and antiemetic drug even for gravidic nausea before being banned due to the teratogenic side effects of one of its two enantiomers. Currently, thalidomide **90** is mainly used for the treatment of certain cancers (multiple myeloma) due to its immunomodulatory properties. The mechanism of action of **90** was fully characterized only recently, demonstrating that its primary target is cereblon, a component of the cullin-dependent ubiquitin ligase responsible for the proteasome-mediated degradation of proteins.^{353–355} Compound **91** (Figure 10) is the result of the conjugation of thalidomide **90** with the pan-BET inhibitor JQ1. Thanks to the capability of its thalidomide portion to recruit cereblon, **91** selectively increased the degradation of BRD2, BRD3 and BRD4 inducing higher apoptosis than JQ1 in leukemia cell lines.³⁵⁶ Compound **92** (Figure 10) displayed a longer linker between thalidomide and JQ1 portions and led to fast, efficient, and prolonged degradation of BRD4 in all the tested Burkitt's lymphoma cell lines.³⁵⁷

Many other examples of PROTACs built with epigenetic ligands have been reviewed very recently.³⁵⁸

4.6. Dual Inhibitors Hitting Two or More Epigenetic Targets

All the examples discussed so far are the results of conjugation of two active substances in which only one portion has an epigenetic target. Here, we are going to consider series of multifunctional compounds in which both fragments interact with two (or more) epigenetic targets.

LSD1 and JmjC KDM4A/C enzymes play a key role in human prostate cancer because they are co-expressed and co-localized with AR.³⁵⁹ We designed and synthesized some LSD1/JmjC hybrid molecules³⁶⁰ by coupling the skeleton of the well-known LSD1 inhibitor

tranylcypromine **24**, to the carboxylate group of either the 4-carboxy-4'-carbomethoxy-2,2'-bipyridine **93**³⁶¹ (Figure 11) or the 5-carboxy-8-hydroxyquinoline (5-carboxy-8-HQ) **94**³⁶² (Figure 11), two 2-oxoglutarate competitive scaffolds developed as JmjC inhibitors. In the two hybrid compounds **95** and **96** (Figure 11), the common tranylcypromine portion makes a covalent adduct with FAD to inhibit LSD1, while the carboxylic acid portion released from the bipyridyl prodrug **95**, as well as the hydroxyquinoline group of **96**, can chelate the JmjC protein-bound iron leading to enzyme inhibition. In cellular assays, **95** and **96** increased H3K4 and H3K9 methylation levels producing growth arrest and apoptosis in LNCaP prostate and HCT116 colon cancer cells. Most importantly, when tested in mesenchymal progenitor (MePR) non-cancer cells, **95** and **96** induced little or no apoptosis, showing a characteristic cancer-selective action.

The KMT G9a is a H3K9 methyltransferase which leads to gene silencing and is overexpressed in different types of tumors. Recent studies demonstrated that the inhibition of G9a decreases cancer cell proliferation, blocks metastases and delays tumor progression.^{363,364} Our researches on properly substituted quinazoline structures allowed us to identify novel H3K9 methyltransferase/demethylase as well as DNMT3A inhibitors bearing a quinazoline structure.^{365,366} In particular, structural manipulations of BIX01294 **97**³⁶⁷ (Figure 11) and UNC0638 **98**³⁶⁸ (Figure 11), two G9a inhibitors largely known in literature, led us to the identification of MC3774 **99** (Figure 11), a Lys-mimicking derivative displaying dual G9a/LSD1 inhibition.³⁶⁹ MC3774 **99** is characterized by a dimethylaminopropylamine chain at C2, a 4-aminobenzylpiperidine moiety at C4 and two methoxy groups at C6 and C7 positions of the quinazoline scaffold. We designed **99** supposing that the C2-propanamine moiety could mimic the H3K4me2 skeleton of the substrate bound within the LSD1 active site. Surprisingly, the compound does not enter into the LSD1 catalytic tunnel but binds the enzyme placing five copies of its molecule in a stacked way obstructing the entrance of the LSD1 active site. The orientation of the molecules can be “face to face” or “head to tail”, and in both modes, they interact with a cluster of negatively charged amino acidic residues.³⁶⁹ This kind of non-covalent and reversible inhibition is typical for the interaction with LSD1, because **99** does not inhibit G9a using the same stacking mode. In MV4–11 leukemia cells, **99** showed anti-proliferative activity with IC₅₀ = 0.89 μM, comparable with the potency observed against LSD1 and G9a at the enzyme level.³⁶⁹

G9a is known to physically interact with DNMT1 to coordinate histone and DNA methylation during cell division,³⁷⁰ promoting repression of target gene expression. From these findings, a global reduction of DNA as well as H3K9 methylation can lead to reactivation of tumor suppressor genes, inhibition of cancer cell proliferation, arrest of cell cycle and induction of apoptosis.^{371,372} The crystal structure of the UNC0638/G9a complex revealed that the C4 aminopiperidine moiety of the ligand as well as the C7 pyrrolidinopropoxy chain are important for specific interactions in the active site of the enzyme, and that, most importantly, while the protonated (at physiological pH) N1 of the quinazoline interacts, through a salt bridge, with Asp1088, the quinazoline N3 did not take part in any direct and crucial interaction with G9a.³⁶⁸ Thus, Rabal *et al.* decided to design and synthesize a quinoline analog of UNC0638 **98**, compound **100** (Figure 11), to be tested

against both DNMT1 and G9a through time-resolved fluorescence resonance energy transfer (TR-FRET) technique. Competition experiments confirmed that **100** displayed substrate-competitive inhibition of both targets, with global reduction in H3K9me2 and 5-methyl cytosine (5mC) levels.^{373,374} Unfortunately, **100** did not show any anti-proliferative response against ALL CEMO-1 ($GI_{50} > 10 \mu M$), DCLBL OCI-Ly3 and OCI-Ly10 (0% inhibition at 1 μM) and AML MV4-11 ($GI_{50} > 10 \mu M$) cell lines. SAR studies focused on the replacement of the C2 substituent and on the *iso*-propyl to methyl change at the piperidine moiety led to CM-272 **101** (Figure 11), with improved potency against DNMT1 and displaying antiproliferative activities in cells ($GI_{50} < 1 \mu M$ against the above reported cell lines) joined to low CYP450 isoforms inhibition, good stability in human microsomes, acceptable exposure and good half-life after intravenously administration. Importantly, treatment with **101** induced anticancer effects *in vivo* against ALL, AML and diffuse large B-cell lymphoma (DLBCL) xenogeneic models.³⁷⁵

Hepatocellular, pulmonary, prostatic carcinoma and leukemia are associated with a global histone deacetylation state and with the double methylation of H3K9.^{376–378} From these evidences, it emerged the need to design inhibitors capable to interact with both G9a and HDAC enzymes. Merging the quinazoline scaffold typical of BIX01294 **97** with polymethylene hydroxamate chains allowed the identification of dual G9a/HDAC inhibitors. Among them, **102** (Figure 11) showed low toxicity, good physico-chemical properties, and antiproliferative activity in the micromolar range in HeLa and K562 cell lines.^{379,380}

As previously discussed, G9a plays a key role in melanoma, breast and lung cancer mediating cellular growth and regulating cell cycle and metabolism pathways.³⁸¹ Another KMT, the H3K27 methyltransferase EZH2, has a repressive role in gene expression, and has been found mutated in B-cell lymphoma and upregulated in breast and prostate carcinoma.³⁸² Simultaneous inhibition of G9a and EZH2 exerted synergistic effects in MDA-MB-231 TNBC.³⁸³ Compounds like **103** (Figure 11), strictly related to BIX-01294 **97**, showed dual G9a/EZH2 inhibition with decrease of both H3K9me2 and H3K27me3 levels and upregulation of genes typically repressed by EZH2 (JMJD3, KRT17, FBX032). Importantly, these dual G9a/EZH2 inhibitors displayed growth arrest in a panel of breast cancer and lymphoma cell lines with low to sub-micromolar IC_{50} values.³⁸³

Curcumin **104** (Figure 11) exerts its powerful anticancer activities with several mechanisms of action, some of them involving epigenetic targets, such as the regulation of HDAC, HAT, DNMT1 and miRNA activities.³⁸⁴ Structurally related to curcumin **104**, some unsaturated (di)ketones were reported as epigenetic multiple ligands.³⁸⁵ With appropriate substitutions at the phenyl rings, these compounds (such as **105–108**, Figure 11) could act as simultaneous inhibitors of SIRT, HAT, KMT and PRMT.³⁸⁵ When tested in human leukemia U937 cells, **105–108** displayed higher apoptosis, and **105** and **106** increased the cell differentiation percentage respect to the corresponding single-target inhibitors.³⁸⁵

A screening of related chromatin modulating compounds allowed Petraglia *et al.* to identify MC2884 **109** (Figure 11), a novel dual HAT/EZH2 micromolar inhibitor (IC_{50} p300 = 45 μM , % inhibition of EZH2 40% and 80% at 5–25 μM , respectively) able to induce the decrease of H3K27me3, H3K9/14ac and H3K27ac levels as well as the induction of

caspase-dependent apoptosis associated with a strong, dose-dependent and specific anticancer activity in several cancer cell lines (in particular leukemia cells) and a good safety profile (mesenchymal progenitor model MePR2B and endometrial stromal cells were non-responsive to treatment). In addition, cellular analysis revealed that MC2884 **109** displayed a synergistic effect on cancer cells death, while no effect was registered when the p300 inhibitor C646 or the EZH2 inhibitor GSK126 were used alone or in combination.

The marked apoptotic effect is due to its capability to induce the modulation of specific mitochondrial pathways of cell death, in addition to the reduction of acetylation of gene promoter regions, downregulating the expression of different anti-apoptotic factors such as BCLX(L) and BCL2. The high anticancer activity of MC2884 **109** against hematological, solid and aggressive models of cancer (TET2^{-/-} and p53^{-/-}) and *in vivo* xenograft models (colon cancer, AML and APL), as well as its massive apoptosis induced in *ex vivo* human primary leukemia blasts with poor prognosis *in vivo*, affords a new model of personalized precision medicine by coupling the use of epi-drugs with epigenome analysis.

UVI5008 **111** (Figure 11) was obtained by chemical substitution of the aromatic portion of the dimeric natural product psammaphin A **110** (Figure 11), known as HDACs (in particular HDAC1–4) and DNMT inhibitor.³⁸⁶ UVI5008 **111** showed inhibition on the same epigenetic targets as **110** plus sirtuins,³⁸⁷ and evoked apoptotic effects in several human cancers such as leukemia, osteosarcoma, melanoma, breast, prostate and colon carcinoma.

DNMT and HDAC are known to work synergistically to induce tumor suppressor gene silencing. This cross talk between DNA methylation and histone (de)acetylation is essential for the control of gene expression, and its dysregulation contributes to the development of cancer.^{388–390} Interesting evidences showed that the simultaneous inhibition of HDAC and DNMT had a synergistic effect in some types of cancers, encouraging the design of dual inhibitors for these two classes of enzymes.^{388,389,391–393} A series of inhibitors based on the structure of the compound NSC-319745³⁹⁴ have been synthesized, by changing the substitutions at the benzamide portion and by converting the carboxylic acid function into the corresponding hydroxamate. Compound **112** (Figure 11) displayed higher DNMT1 inhibition than NSC-319745 and inhibited HDAC1 and HDAC6 at nanomolar levels. In cellular assays, **112** (Figure 11) showed effective cytotoxicity against human K562 and U937 cancer cells, with low micromolar IC₅₀ values. In particular, in U937 cells, **112** increased the H3K9 and H4K8 acetylation levels, upregulated p16 expression by *p16* CpG islands demethylation, and induced remarkable apoptosis.³⁹⁵

Since HDACs and LSD1 are overexpressed in several types of human cancers contributing to the silencing of tumor suppressor genes, also the simultaneous inhibition of these two enzymes can furnish synergistic activity against cancer cell growth, migration and invasion. The LSD1 inhibitors ORY-1001 **25** and GSK2879552 **26**, currently in clinical trials for the treatment of leukemia, demonstrated that the amino group of tranylcypromine **24** can be substituted without loss of LSD1 inhibiting activity. Thus, compound **113** (Figure 11) was designed, prepared and tested by introducing the vorinostat **3** structure into the amino group of tranylcypromine **24**. Compound **113** inhibited LSD1 at micromolar and HDAC1/2 at nanomolar levels and showed antiproliferative activities higher than vorinostat **3** against

A549 lung, MCF-7 breast, MGC-803 gastric, and SW-620 colorectal cancer cells. Target engagement was demonstrated in MGC-803 cells, where **113** increased methyl-H3K4/methyl-H3K9 together with acetyl-H3 levels, decreased the mitochondrial membrane potential, and induced apoptosis.³⁹⁶

Our contribution in this field was to prepare novel LSD1/HDAC dual inhibitors by introduction of the HDAC inhibiting hydroxamate or benzamide side chain at the phenyl ring of tranylcypromine **24**, where we previously inserted various amide or amino acid substituents that highly improved the LSD1 inhibitory potency of **24** itself.^{30,397–401} Size exclusion chromatography revealed stable association of the HDAC1:LSD1:CoREST ternary complex with 1:1:1 stoichiometry. The most potent compound, **114** (Figure 11), bound the CoREST ternary complex showing both LSD1 and HDAC1 inhibition at submicromolar level, and exhibited higher antiproliferative activity than entinostat **9** against cutaneous squamous carcinoma and several melanoma cell lines, being less toxic than **9** towards keratinocytes and melanocytes. Importantly, **114** slowed down tumor growth in a melanoma mouse xenograft model.⁴⁰²

Milelli *et al.*⁴⁰³ developed a series of polyamine-based hybrid compounds, from which emerged **115** (Figure 11), showing interesting inhibitory activity against LSD1 (IC₅₀ LSD1-CoREST3= 3.8 μM) and HDAC1 (Ki HDAC1-CoREST3= 42.5 nM) in *vitro* as well as in cellular assays. In **115**, the two anti-HDAC and -LSD1 pharmacophoric portions were connected using a polyamine-type chain, since the polyamine moiety can interact with both LSD1 and HDAC enzymes^{404–406} due to protonated nitrogen atoms allowing electrostatic interactions with negatively charged amino acid residues.^{407,408} Among the different polyamine linkers used, the spermine (3-4-3), typical of **115**, produced the best enzymatic results. When tested against MCF7 breast cancer cell line, **115** resulted more effective (at high concentration) than vorinostat **3** in inducing cytotoxicity (68.6% vs 56.6% of dead cells).⁴⁰³

Design and synthesis of dual inhibitors of BRDs and HDACs arises from the evidence that both these families of proteins take part in the acetylation process, and the independent inhibition of each single target is connected with similar genetic and biological effects.^{409,410} The 9H-purine scaffold has been reported to bind with different strength the BRD proteins,⁴¹¹ and some N⁶-benzoyladenine derivatives have been described as BRD4 inhibitors.⁴¹² In these last compounds, the N⁶-benzamido group mimics the acetyl-lysine substrate in the binding with BRD4, and electron-donating groups at the C2'-benzamide position are preferred to obtain high inhibition. Thus, compound **116** (Figure 11) was designed, among others, as a dual BRD4/HDAC inhibitor by incorporating the polymethylene hydroxamate chain typical of vorinostat **3** into the C2'-benzamide position of the N⁶-benzoyladenine. In HL-60 cells, **116** arrested cell proliferation, induced apoptosis, enhanced the ATRA-induced differentiation and inhibited c-MYC production. Importantly, **116** exhibited growth arrest in cells resistant to BRD4 inhibitors.⁴¹³ The 3,5-dimethylisoxazole is another substructure able to mimic acetyl-lysine in the binding with BRDs.⁴¹⁴ The introduction of the N-hydroxyheptanamide substituent at the 3-benzoyl-5-(3,5-dimethylisoxazol-4-yl)phenoxy moiety of a BRD4 inhibitor furnished to the new molecule **117** (Figure 11) dual activity against both BRD4 and HDAC1 at submicromolar level.

Compound **117** showed antiproliferative activities against AML, MV4–11, and chronic myelogenous leukemia (CML) K562 cell lines. Docking studies performed on **117** confirmed what previously explored by X-ray studies on the 3,5-dimethylisoxazole, revealing an interesting complementarity of the compound with the binding pocket of BRD4, and showing a binding into the HDAC1 pocket similar as that of vorinostat **3**.⁴¹⁵

5. Conclusions and future perspectives

MMT, MCM and MTDLs seem to be very favorable approaches for the treatment of complex diseases. Indeed, to date, it is well known that quite often single agent-based therapy characterized by a selective mechanism of action due to the engagement of a specific target is not enough, especially in multifactorial diseases. Despite the success of MMT at pre-clinical and clinical stage, the potential formulation problems, drug-drug interactions and the consequence toxicity lead us and others to hypothesize that the MTDL approach could be, even if not always, a true promise of polypharmacology.

In fact, the potential development of drugs that can be defined as “intelligent” or rather able to induce the inhibition or the degradation (as it happens for PROTACs) of carefully chosen targets, could turn out to be the true frontier of epigenetic and non-epigenetic research. Specifically, the role of the epigenetic cross-talk mechanisms associated with different types of dysregulation is well-accepted in different complex diseases such as cancer, neurodegenerative and metabolic disorders. Therefore, despite the frequent difficulty in structural optimization in order to effectively merge the pharmacophoric portions that characterized the entities of interest, the development of hybrid drugs able to simultaneously hit two or more targets in which one (or more) is (are) epigenetic and the other(s) belongs to a non-epigenetic category(ies) results very promising, as seen for dual HDAC/TK inhibitors, for which the two compounds CUDC-101 **88** and CUDC-907 **89** have already entered clinical trials. In addition, another important challenge which can be won through the polypharmacology approach is to extend the scope of epigenetic drugs beyond the limited spectrum of hematologic cancers, the only ones for which the epi-drugs have been approved so far.

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ABBREVIATIONS USED

AD	Alzheimer disease
AhR	aryl hydrocarbon receptor
AKT	protein kinase B
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
AR	androgen receptor
ATC	anaplastic thyroid cancer
ATRA	all- <i>trans</i> retinoic acid
BBB	blood brain barrier
BCL2	B-cell lymphoma 2
BCL2i	B-cell lymphoma 2 inhibitors

BDNF	brain derived neurotropic factor
BET	bromo and extra terminal
BRD	bromodomain
CDCK4i	CDK4 inhibitors
CDCKS	cyclin-dependent kinases
cGMP	cyclic guanosine monophosphate
CLL	chronic lymphocytic leukemia
CREB	cAMP response element
CRPC	castration-resistant prostate cancer
CTA	cancer testis antigen
CTCL	cutaneous T-cell lymphoma
DNMTi	DNMT inhibitors
DNMTs	DNA methyltransferases
DOT1L	disruptor of telomeric silencing 1-like
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
EZH2	enhancer of zeste homolog 2
FACS	fluorescence-activated cell sorting
FAD	flavin adenine dinucleotide
FDA	food and drug administration
FGF	fibroblast growth factor
FLT3	FMS-related tyrosine kinase 3
GO	gentuzumab ozagamicin
HATs	histone acetyltransferases
HCC	hepatocellular carcinoma
HDACi	HDAC inhibitors
HDACs	histone deacetylase
HER2	human epidermal growth factor receptor 2

HER3	human epidermal growth factor receptor 3
HMT	histone methyltransferases
8-HQ	8-hydroxyquinoline
HSP90	heat-shock protein 90
IDO1	indoleamine-2,3-dioxygenase
ITP	inhibition of tubulin polymerization
JAK1	janus kinase 1
JmjC	Jumonji C
KDM	lysine demethylase
KLF4	kruppel like factor
KMTs	lysine methyltransferases
KP	kynurenine pathway
LSD1	lysine-specific histone demethylase 1
LSD2	lysine-specific histone demethylase 2
MAOs	monoamine oxidases
MAOi	MAO inhibitors
MAPK	mitogen activated protein kinase
MBD	methyl binding domains
MCM	multi-compound medication
MDS	myelodysplastic syndrome
MePR	mesenchymal progenitor
MET	hepatocyte growth factor receptor
MLL	mixed-lineage-leukemia
MM	multiple myeloma
MMT	multiple-medication therapy
MPM	malignant pleural mesothelioma
MTDL	multi-target-directed ligand
mTOR	mammalian target of rapamycin
NAD⁺	nicotinamide adenine dinucleotide

NAMPT	nicotinamide phosphoribosyltransferase
NAMPTi	nicotinamide phosphoribosyltransferase inhibitors
NFK	<i>N</i> -formylkynureine
NRAS^{Mut}	NRAS-mutant
NSCLC	small cell lung carcinoma
PD	pharmacodynamics
PDE5	phosphodiesterase 5
PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidylinositol-3-kinase
PI3K/Akt/mTOR	phosphatidylinositol-3-kinase
PK	pharmacokinetics
PML	promyelocytic leukemia gene
PRMT	arginine methyltransferase
PROTAC	proteolysis targeting chimera
pTau	tau protein
PTCL	peripheral T-cell lymphoma
RAR-β	retinoic acid receptor beta
RARE	retinoic acid responsive elements
SAHA	suberoylanilide hydroxamic acid
SAM	<i>S</i> -adenosylmethionine
SAR	structure-activity relationship
STS	soft tissue sarcoma
TG	transglutaminase
TG2i	TG2 inhibitors
TKi	tyrosine kinase inhibitors
TNBC	triple negative breast cancer
TR-FRET	fluorescence resonance energy transfer
UR	urothelial carcinoma
VEGF	vascular endothelial growth facto

VEGFR	vascular endothelial growth factor receptor
VPA	sodium valproate
wt	wild type

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Figure 1.
The first clinical success of the MMT based on the co-administration of methotrexate **30**, vincristine **31**, prednisone **32** and 6-mercaptopurine **33** for ALL treatment.

Figure 2. Combinations between HDACi and DNMTi (A, B, and D), and between HDACi, ATRA and doxorubicin (C).

Figure 3.

Combinations between genistein with broccoli sprouts (E), HDACi with platinum complexes (F), BETi with MEKi (G), or with HDACi (H-J), or with BCL2i (J).

Figure 4.

Combinations of HDACi and EZH2i (K), DNMTi with a conventional anticancer agent (L), or with monoclonal antibody (N), or with BCL2i and eventually antifungal agent (P, Q), HDACi with monoclonal antibody plus conventional anticancer agents (M), HDACi plus antiandrogen anticancer agent (O).

Figure 5. Rational design of the HDAC/TKi **CUDC-101** and **CUDC-907**, in clinical trial for the treatment of cancer. IC₅₀ values for the different targets are indicated, when available. The HDAC inhibitory portions are depicted in red.

Figure 6.

Structures of novel HDAC/TK inhibitors based on structures of PI3K, FGFR, CDK (ribociclib **59** and abemaciclib **61**), VEGFR (pazopanib **63**), JAK (ruxolitinib **66**)/HSP90 (BEP800, **67**) inhibitors. IC₅₀ values are indicated, when available. The HDAC inhibitory portions are depicted in red. The HSP90 inhibitory portion is depicted in blue.

69**osimertinib****EGFR** 0.48 μM **LSD1** 3.8 μM **Figure 7.**Structure of osimertinib **69**, a dual EGFR/LSD1 inhibitor.

70**SB284851-BT**

BRDT (1)	18 μ M
BRD4 (1)	3.7 μ M
BRDT (2)	> 250 μ M
BRD4 (2)	ND

71

BRDT (1)	3.5 μ M
BRD4 (1)	1.7 μ M
BRDT (2)	>250 μ M
BRD4 (2)	>250 μ M
BRD3 (1)	11 μ M
BRD3 (2)	> 100 μ M
BRD2 (1)	29 μ M
BRD2 (2)	67 μ M
K_d p38α	0.47 nM

Figure 8.
Structure of dual BET/TK inhibitors.

Figure 9.

Structures of ATRA-based HDACi, dual HDAC/IDO1 inhibitors, dual HDAC/NAMPT inhibitors, lefloxacin- and *iso*-combretastatin A-4-based HDACi, dual HDAC/HSP90 inhibitors, ebselen-based HDACi, dual HDAC/PDE5A inhibitors, HDAC/TG2 inhibitors, dual HDACi/NO donor compound. IC₅₀ values are indicated, where available. The HDAC inhibitory portions are depicted in red. The HSP90 inhibitory portion is depicted in blue.

Figure 10.
Structures of BET/PROTAC compounds.

Figure 11.

Structure of pan-KDMs inhibitors, dual G9a/LSD1 inhibitors, dual G9a/DNMT inhibitors, dual G9a/HDAC inhibitors, dual G9a/EZH2 inhibitors, multi-target inhibitors, dual HAT/EZH2, dual DNMT/HDAC inhibitors, DNMT/HDAC/SIRT inhibitor, dual DNMT/HDAC, dual LSD1/HDAC inhibitors and dual BRD4/HDAC inhibitors. IC₅₀ values are indicated, where available. The HDAC inhibitory portion is depicted in red.

Table 1.

Clinically approved and clinical candidates among epigenetic modulators.

Compound	Structure	Epi-Target	Clinical implication	Status	Clinical trial number(s)
1 Azacytidine		DNMT	acute myeloid leukemia, myelodysplastic syndrome	FDA-approved, 2004	
2 Decitabine		DNMT	acute myeloid leukemia, myelodysplastic syndrome	FDA-approved, 2006	
3 Vorinostat		HDAC	cutaneous T-cell lymphoma	FDA-approved, 2006	
4 Romidepsin		HDAC	cutaneous T-cell lymphoma	FDA-approved, 2009	
5 Belinostat		HDAC	peripheral T-cell lymphoma	FDA-approved, 2014	
6 Panobinostat		HDAC	multiple myeloma	FDA-approved, 2015	
7 Tucidinostat (Chidamide)		HDAC	peripheral T-cell lymphoma	Chinese FDA-approved, 2015	
8 Sodium Valproate		HDAC	progressive, non-metastatic prostate cancer breast cancer solid tumors	Phase I/II	

Compound	Structure	Epi-Target	Clinical implication	Status	Clinical trial number(s)
9 Entinostat		HDAC	breast cancer invasive breast cancer ER-negative, PR-negative, HER2-negative (triple negative) breast cancer	Phase I/II	
10 Mocetinostat		HDAC	solid tumors, Hodgkin's and non-Hodgkin's lymphoma, leukemia	Phase I/II	
11 Abexinostat		HDAC	Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, leukemia, lymphocytic B-cell lymphoma	Phase I/II	
12 Pracinostat		HDAC	solid tumors, hematologic malignancies, myelodysplastic syndrome	Phase I orphan drug for AML	
13 Quisinostat		HDAC	cutaneous T-cell lymphoma	Phase I/II	
14 Resminostat		HDAC	advanced colorectal carcinoma	Phase I/II orphan drug for hepatocellular carcinoma	
15 Givinostat		HDAC	myeloproliferative diseases, Hodgkin's lymphoma	Phase I/II Orphan drug for Duchenne muscular dystrophy	
16 Rocilinostat		HDAC6	lymphoma, lymphoid malignancies, multiple myeloma	Phase I/II	
17 Nicotinamide		Sirtuins	solid tumors	Phase II/III	
18 GSK2816126		EZH2	acute myeloid leukemia, non-Hodgkin lymphoma, multiple myeloma	Phase I/II	
19 Tazemetostat		EZH2	different kinds of lymphomas and solid tumors	Phase I/II	

Compound	Structure	Epi-Target	Clinical implication	Status	Clinical trial number(s)
20 CPI-1205		EZH2	B-cell lymphoma	Phase I	
21 Pinometostat		DOT1L	mixed-lineage leukemia	Phase I	*
22 JNJ-64619178		PRMT5	relapsed/refractory B cell non-Hodgkin lymphoma, advanced solid tumors	Phase I	
23 GSK3326595		PRMT5	selected solid tumors and non-Hodgkin's lymphomas, neoplasms	Phase I	
24 Tranylcypromine		LSD1	acute myelogenous leukemia	Phase I/II	
25 ORY-1001		LSD1	myeloid leukemia, small cell lung cancer, relapsed or refractory acute leukemia	Phase I/II	UDRACT n° 2013-002447-29 2018-000482-36 2018-000469-35
26 GSK2879552		LSD1	myelodysplastic syndrome leukemia small cell lung carcinoma	Phase II/I	
27 CPI-0610		BRD family	multiple myeloma, lymphoma	Phase I	
28 GSK525762		BRD family	relapsed refractory hematological malignancies, midline carcinoma	Phase I	
29 OTX015		BRD family	glioblastoma multiforme, triple negative breast cancer and other solid tumors, acute leukemia	Phase II/I	

* Only in combination with azacytidine **1** or cytarabine, daunorubicin hydrochloride and pinometostat **21**.

** Only in combination with tretinoin or all-*trans* retinoic acid and cytarabine.

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