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Toward the Development of Innovative Bifunctional Agents To Induce Differentiation and To Promote Apoptosis in Leukemia: Clinical Candidates and Perspectives

Ioannis S. Vizirianakis,*,† Maria Chatzopoulou,‡ Ioannis D. Bonovolias,† Ioannis Nicolaou,‡ Vassilis J. Demopoulos,‡ and Asterios S. Tsiftsoglou†

†*Laboratory of Pharmacology and* ‡*Laboratory of Pharmaceutical Chemistry, Department of Pharmaceutical Sciences, Aristotle University of Thessaloniki, GR-54124 Thessaloniki, Greece*

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1. Introduction

Although the outcome of therapy for leukemia has improved over the years, mainly in younger patients, less than a third of adults with acute myeloid leukemia (AML[a]), for example, are cured by current treatments, a fact stressing the need for new therapeutic approaches. Since leukemias are considered disorders of self-renewal, differentiation, and apoptosis of hematopoietic stem cells (HSCs) and/or their early progenitors, the treatment of leukemia is rapidly changing from conventional chemotherapy toward a more innovative individualized and targeted therapy.[1,2]

The discovery of leukemia stems cells (LSCs) in the late 1990s[3,4] as a minor fraction within the subpopulation of hematopoietic cells and the compelling research efforts initiated thereafter have clearly shown that many malignancies are maintained via stemlike cells having the capacity for indefinite self-renewal. This LSC hypothesis has established the notion that the emergence of drug resistance and the clinical relapse of leukemias following an initial remission induced by cytotoxic or targeted therapy agents is related to acquired mutations of LSCs. Therefore, eradication of LSCs is considered necessary for the radical treatment of leukemias. As a matter of fact, novel exploitable targets for leukemia therapy emerged including enzymes like tyrosine kinases involved in signal transduction pathways, genes encoding proteins that regulate apoptosis and differentiation of malignant cells, cell-lineage transcriptional factors, angiogenesis factors, and unique proteins driving the cell cycle machinery.[1,2,5−10] Interestingly, within the group of antileukemia agents exist small molecule drugs like tyrosine kinase inhibitors, proteasome inhibitors, farnesyl transferase inhibitors, hypomethylating agents, histone deacetylase inhibitors, mTOR targeting agents, bcl-2 inhibitors, and inhibitors of cyclin-dependent kinases (Figure 1).

This paper is a comprehensive overview of the scientific efforts made to develop novel antileukemia therapeutics by presenting chemical, pharmacological, and pharmacogenomic data obtained during preclinical and clinical assessment of these agents. Furthermore, the designated synthesis of new medicines inducing differentiation, cell cycle arrest, and/or promoting apoptosis along with multitargeted therapeutics will be also discussed. Such novel agents can be used in combination with other agents modulating different signaling pathways and molecular targets within the leukemia cells to overcome the emergence of drug resistance. This information can then be discussed from a pharmacogenomic view of antileukemia therapeutics. Individual genetic variations recorded in antileukemia drug therapy can be critical for personalized medicine and their clinical exploitation can achieve better pharmacotherapy outcomes.

2. Leukemias as Clonal Disorders of HSCs and Early Multipotent Progenitors

Studies in the early 1970s by Fialkow et al. have revealed that human leukemias are more or less clonal disorders originating from self-proliferating hematopoietic cells in the bone marrow.[11] Although myeloblasts, erythroblasts, lymphoblasts, and others of early undifferentiated progenitors were considered responsible for specific types of acute or chronic myeloid, erythroid, and lymphoid leukemias, it was not clear which one of the stem cells and/or early progenitors are involved in leukemogenesis.

*To whom correspondence should be addressed. Phone: +30-2310-997658. Fax: +30-2310-997645. E-mail: ivizir@pharm.auth.gr.

[a] Abbreviations: 5-FU, 5-fluorouracil; 6-MP, 6-mercaptopurine; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; APL, acute promyeloid leukemia; Ara-C, cytarabine (cytosine arabinoside); ATRA, all-trans retinoic acid; Bcl, B-cell lymphoma; CDKs, cyclin-dependent kinases; CENP, centromere protein; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; CRABPs, cellular retinoic acid-binding proteins; CSF-1R, colony stimulating factor 1 receptor; DHFR, dihydrofolate reductase; DMSO, dimethyl sulfoxide; DNMTs, DNA methyltransferases; DZNep, 3-deazaneplanocin A; EGCG, (−)-epigallocatechin 3-gallate; ERK, extracellular signal-regulated protein kinase; FKBP12, FK506-binding protein; FLT3, fms-like tyrosine kinase 3; FPP, farnesyl pyrophosphate; FRB, FKBP-rapamycin-binding domain; FTase, farnesyl transferase; FTI, farnesyl transferase inhibitor; G-CSF, granulocyte-colony stimulating factor; GIST, gastrointestinal stromal tumor; HDACs, histone deacetylases; HDACIs, histone deacetylase inhibitors; HES, hypereosinophilic syndrome; HMBA, hexamethylene bisacetamide; hOCT1, human organic cation transporter 1; HSCs, hematopoietic stem cells; HSP90, heat shock protein 90; IFN, interferon; ITD, internal tandem duplication; LICs, leukemia initiating cells; LSCs, leukemic stem cells; MAPK, mitogen activated protein kinase; MDS, myelodysplastic syndrome; MDR, multidrug resistance; MEL, murine erythroleukemia cells; MM, multiple myeloma; MTHFR, 5,10-methylentetrahydrofolate reductase; mTOR, mammalian target of rapamycin; MTX, methotrexate; NF-κB, nuclear factor κB; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; P-gp, P-glycoprotein; PDGFR, platelet derived growth factor receptor; PEG, polyethylene glycol; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKI, protein kinase inhibitor; PTEN, phosphatase and tensin homologue; RAR, retinoic acid receptor; RBCs, red blood cells; RXR, retinoid X receptor; SAHA, suberoylanilidehydroxamic acid; SAR, structure−activity relation; SBHA, suberic bishydroxamic acid; SMI, small molecule inhibitor; TG, thioguanine; TNF, tumor necrosis factor; TPMT, thiopurine *S*-methyltransferase; TrkA, tropomyosin receptor kinase A; TSA, trichostatin; TYMS, thymidylate synthase; VEGFR, vascular endothelial growth factor receptor; VPA, valproic acid; XIAP, X-linked inhibitor of apoptosis protein.

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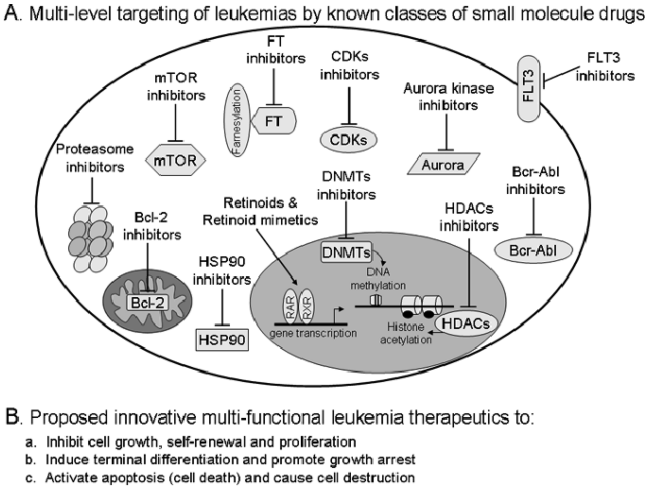

Figure 1. Diagrammatic presentation of molecular targets for known classes of small molecule antileukemia drugs used in clinical practice or under development and the proposed strategy for innovative multifunctional leukemia therapeutics (see text for details). Abbreviations are as follows: HSP, heat shock protein; RAR, retinoid acid receptor; RXR, retinoid X receptor; DNMTs, DNA methyltransferases; HDACs, histone deacetylases; CDKs, cyclin-dependent kinases; mTOR, mammalian target of rapamycin; FT, farnesyl transferase; FLT3, Fms-like tyrosine kinase 3.

The study of experimental leukemias (mouse and human) in culture and the unique ability of certain cell fraction in the leukemic cell population to develop colonies with unrestricted ability to grow via self-renewal made quite clear and also confirmed that leukemias are indeed clonal disorders initiating from a small number of undifferentiated cells. In term of properties, such cells were grown and maturated in vitro (cultures) and in vivo (animals) having a specific neoplastic phenotype relatively stable.[2,10] In contrast to normal hematopoietic cells, leukemic cells remain in culture undifferentiated with an acquired ability to proliferate and invade but unable to senesce.

The evidence that normal hematopoietic cells grown in fetal liver or in spleen can be transformed into leukemia cells able to propagate the leukemia in mice, and also samples of primary leukemic cells derived from leukemic subjects are grown and developed the disease in NOD/SCID mice,[4] leaves no doubt that leukemogenesis is initiated at the level of either HSCs or early uncommitted progenitors. LSCs exhibit the same cell surface antigens with normal HSCs. Leukemias usually are initiated within the bone marrow environment and affect normal hematopoiesis either via paracrine function or otherwise in the stem cell niche.[1] Regardless how LSCs and leukemia-initiating cells (LICs) are born, grow, progress via self-renewal, and intersect with normal HSCs in their microenvironment, their existence indicates that leukemic cell growth and/or self-renewal is regulated at the cellular level via signaling pathways induced by external stimuli and operating at different levels: level of HSC niche, erythroblastic islands, and other sites. SCF/c-kit, Notch, Wnt, and Epo are critical signaling pathways that give the ability to LSCs to propagate the disease. Moreover, epigenetic events or production of fused transcription factors and enzymes also appear to contribute to leukemic cell growth and maintenance.[1,5,12]

3. Conceptual Approaches Exploiting Induction of Differentiation and Apoptosis To Eradicate LSCs

Chemotherapy of human leukemias with the use of conventional antineoplastic agents administered under several combination protocols is accompanied by severe adverse reactions, including myelosuppression, loss of hair, and transient damage of rapidly replicating tissues. This less selective pharmacological approach used for years was based on the principle of "just killing" in any way the leukemia cells using all types of antineoplastic agents (antimetabolites, antitumor antibiotics, alkylating agents, blockers of mitosis, and even organometallic compounds and natural products).

The knowledge accumulated over the past several years on the nature of leukemic cells indicates that such cells can be converted into nondividing growth arrested cells unable to support malignant growth.[2] Moreover, it has been established that apoptosis can also be induced in leukemic cells using pharmaceutical agents and metabolic modulators, like kinase inhibitors. All this understanding taken together with the dependency of leukemia cells on microenvironmental factors and external stimuli suggests that multilevel targeting can be a fruitful approach to eradicate leukemia cells under less harmful conditions to adjacent normal tissues.[1,5,12] A future challenge will then be to design agents that can differentiate leukemia cells into nondividing/nonmalignant growth-arrested cells and/or promote cell death concomitantly or thereafter in leukemia cell differentiated progeny.

Therefore, at least three classes of agents can be developed, as illustrated in Figure 2: (a) agents able to induce terminal cell differentiation and cessation of self-renewal, (b) agents able to exclusively promote apoptosis by acting at intrinsic or extrinsic signaling pathways, and (c) agents having structural domains that can promote both processes concomitantly by regulating gene activation and/or repression processes. Agents belonging to class A can be very potent inducers of leukemia cell differentiation, like retinoids, As_2O_3, potent molecules like suberoylanilide hydroxamic acid (SAHA, **1b**), phenyl acetate, and butyric acid analogues, or other agents like pyridine derivatives, cyclic ureas, and many others reported elsewhere.[2,9] Class B agents that can promote apoptosis can be either targeted antibodies or inhibitors of kinases involved in cell signaling pathways, tumor necrosis factor (TNF) and TRAIL-like agents as well as BH-mimetics, blocking the function of Bcl protein in mitochondria.[5,6] Agents of class A and class B can be combined appropriately, used either sequentially or in parallel, as depicted for class C_1. Although these agents may differ in their optimum inducing concentration in promoting differentiation and/or apoptosis, pharmacokinetics studies may provide useful and effective management protocols for allowing them to carry out both functions. The most challenging approach, however, has been the design and development of agents of class C_2. Hybrid agents sharing structural domains responsible for promoting differentiation induction and apoptosis in the same leukemia cells can be useful agents to disrupt their functions. The latter class of pharmaceutical agents must regulate critical steps involved in epigenetic regulation of gene activation and silencing. This can be done either via modulation of trans- and cis-elements of superfine structure of chromatin by interacting either directly on the genome at specific promoter sequences or distant elements or via cofactors regulating the active and repressive transcription complexes. Such innovative antileukemic agents can be combined even with relatively lower concentrations of conventional antineoplastic agents.

4. Human Leukemia Cells Can Be Converted into Differentiated Nondividing Progeny

The discovery made by Friend et al. (1971)[13] that virus-transformed hematopoietic progenitors derived from mouse

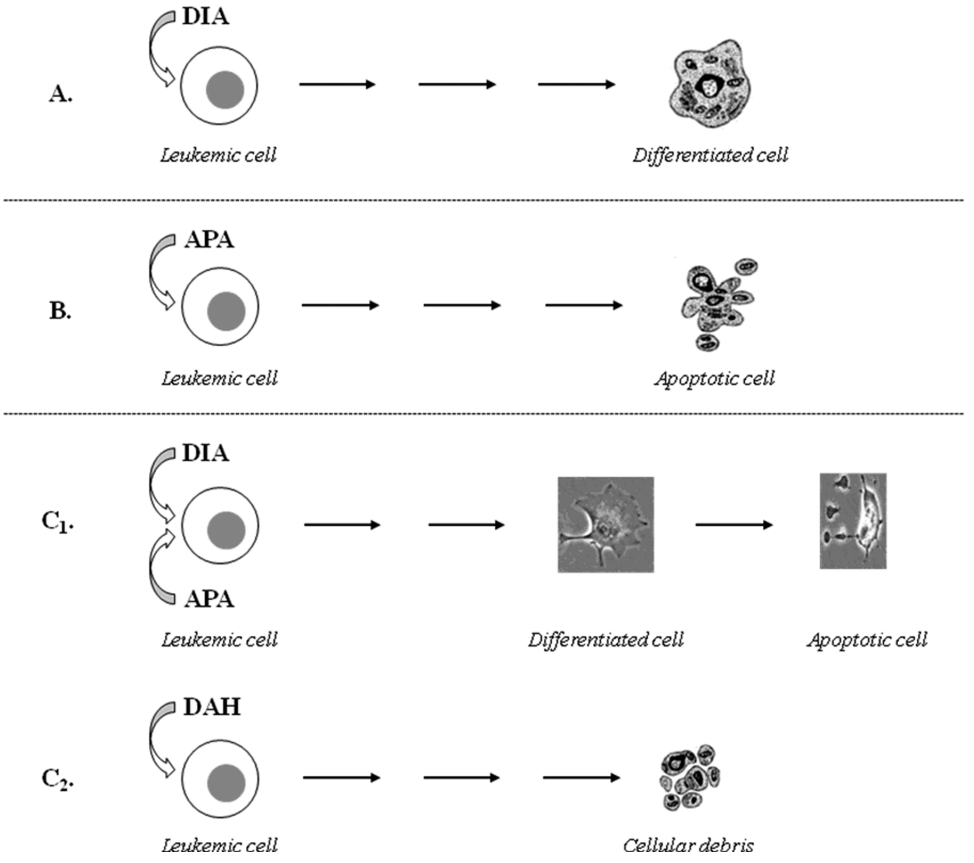

Figure 2. Conceptual approaches combining induction of differentiation and promotion of apoptosis (cell death) of leukemia cells. Differentiation inducing agents (DIA) can disrupt self-renewal and cause growth arrest in terminal differentiated progeny unable to support malignant growth (A and C_1). In contrast, apoptosis promoting agents (APA) can stimulate cell death signaling in undifferentiated as well as differentiated progenitors of leukemic cells (B and C_1). Finally, differentiation and apoptosis hybrid (DAH) agents represent molecules with structural domains as bifunctional agents that can promote differentiation and/or apoptosis leading to eradication of leukemic cells (cell death) (C_2) (see text for details).

spleen cells can be differentiated into hemoglobin producing cells in culture created the fertile ground and sparked many differentiation-related studies in oncology with mouse or human experimental leukemias. Differentiation of many leukemia cell lines (MEL, K-562, HEL, HL-60, KJ1, just to mention few) confirmed the working hypothesis proposed by L. Sachs that leukemic cells still retain the ability to differentiate via epigenetic regulation, regardless of the existing genetic abnormalities in such cells. Conversion of highly malignant leukemia cells into growth-arrested and nondividing progenies (myeloid or erythroid) provided the frame for the so-called "differentiation therapy of cancer", a field that recently began to yield interesting antileukemia therapeutics for the treatment of PML, myelodysplastic syndrome (MDS), and other malignancies.[2,14]

Over the past 30 years, a lot of innovations were made by using in vitro models of leukemia cell differentiation. Such systems have been reviewed elsewhere.[2] In all these cases, the induction of leukemic cell differentiation was achieved in parallel with the development of suitable inducing agents and/or metabolic inhibitors also capable of restricting cell growth of these cells (Table 1). Among the various agents developed, either from high-throughput screening or selection based on predictive structure−activity relations (SAR) methodology, are agents likely to act at specific cell targets, such as metabolic enzymes, retinoid receptors, epigenetic regulators (transcription factors and cis-regulatory elements), and/or proteins with as yet unknown mechanism(s) of action. It is emphasized that the agents developed so far as potent inducers of leukemic cell differentiation using the MEL, HL-60, or K-562 cell model systems are quite diverse in structure and physicochemical properties because of targeting of various cellular components. The site of action also varies from cell membrane sites, signaling pathways, cell cycle kinases, cytoplasmic proteins, nuclear receptors, and several other components.[2] The development of these inducers thus far gave several classes of low molecular weight agents, as shown in Table 1. Some of these agents are cytotoxic on a molecular basis, and others are potent inducers of differentiation that cause cell growth arrest. This depends on their optimum concentration; some of them are nonspecific in causing cell-cycle arrest and cell death, while other agents do the opposite. However, an important issue is the development of bi- or multifunctional agents acting at multiple levels and capable of eradicating the population of LSCs, quiescent or not efficiently. By developing agents that carry two or three functional pharmacophore groups in their molecular structures to modulate different target molecules, one should expect to see better antileukemic agents with improved clinical outcome (Figures 1 and 2).

5. Differentiation Inducing Agents

The discovery of Charlotte Friend established the notion that induction of differentiation by chemical agents in leukemic cells could reprogram the cells toward proliferation arrest and/or programmed cell death.[1,13,15] The first experimental attempts, as initial basic research focused on chemical inducers of differentiation, drew attention to "polar/apolar compounds", later known as hybrid polar compounds. Structure optimization of hexamethylene bisacetamide (HMBA, **1a**) led

Table 1. Chemical Inducers Triggering in Vitro Differentiation of Leukemia Cell Models[a]

inducer	cell model	terminal differentiated phenotype
1-α,25-dihydroxyvitamin D_3	HL-60	monocytic/ macrophage
3-deazauridine	HL-60	granulocytic
5-azacytidine (**2a**)	MEL	erythrocytic
	HL-60	granulocytic
	K-56HL-602	erythrocytic
5-FU	K-562	erythrocytic
6-TG	HL-60	granulocytic
actinomycin D	MEL	erythrocytic
activin A	MEL	erythrocytic
antifolates	HL-60	granulocytic
anthracyclins	HL-60	granulocytic
aphidicolin	HL-60	megakaryocytic
apicidin	K-562	erythrocytic
Ara-C	HL-60	monocytic
	K-562	erythrocytic
benzodiazepines	MEL	erythrocytic
bis-hydroxamic acids	MEL	erythrocytic
	HL-60	granulocytic
butyric acid	HL-60	monocytic
	K-562	erythrocytic
bryostatin-1	HL-60	monocytic
camptothecin	HL-60	granulocytic
chromomycin	K-562	erythrocytic
cis-platin	K-562	erythrocytic
cyclic ureas and thioureas	MEL	erythrocytic
dbcAMP	HL-60	granulocytic
DMSO	MEL	erythrocytic
	HL-60	granulocytic
herbimycin	K-562	erythrocytic
hemin	K-562	erythrocytic
HMBA (**1a**)	MEL	erythrocytic
	HL-60	granulocytic
	K-562	erythrocytic/ megakaryocytic
hydroxyurea	K-562	erythrocytic
hypoxanthine	MEL	erythrocytic
	HL-60	granulocytic
mithramycin	K-562	erythrocytic
phenyl acetate (PA)	K-562	erythrocytic
phorbol esters (PMA, TPA)	HL-60	monocytic/ macrophage
	K-562	megakaryocytic
PMEA	K-562	erythrocytic
purine analogues	MEL	erythrocytic
pyridine derivatives	MEL	erythrocytic
resveratrol	K-562	erythrocytic
retinoids	HL-60	granulocytic
SAHA (**1b**)	MEL	erythrocytic
sodium butyrate	MEL	erythrocytic
	K-562	erythrocytic/ megakaryocytic
tallimustine	K-562	erythrocytic
tiazofurin	HL-60	erythrocytic
	K-562	granulocytic
TSA	MEL	erythrocytic
tunicamycin	HL-60	monocytic
UDP	MEL	erythrocytic
bitamin B_{12}	K-562	erythrocytic
xylosyladenosine	MEL	erythrocytic

[a] For details see ref 2. Abbreviations: Ara-C, cytarabine (cytosine arabinoside); DMSO, dimethyl sulfoxide; 5-FU, 5-fluorouracil; HMBA, hexamethylene bisacetamide; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; SAHA, suberoylanilide hydroxamic acid; 6-TG, 6-thioguanine; TSA, trichostatin; UDP, ureido derivatives of pyridine.

to the discovery of **1b**, a histone deacetylase inhibitor (HDACI). Experimentation with cytotoxic nucleoside analogues implicated in epigenetic regulation of gene expression, along with histone acetylation and methylation, revealed that compounds such as 5-azacytidine[16] (**2a**) and 5-deoxyazacytidine[17] (decitabine, **2b**) induce differentiation of leukemic cells in lower concentrations, with parallel inhibition of DNA methylation via inhibition of the enzyme DNA methyltransferase (DNMT). Finally, induction of differentiation of teratocarcinoma cells by retinoic acid[18] and discovery of retinoic acid receptors[19] revealed its regulatory role in key signaling pathways and its successful clinical application to treat acute promyelocytic leukemia (APL). These three classes of agents are the most extensively studied in the search of chemical inducers of differentiation of leukemic cells and are more thoroughly presented below.

5.1. Hybrid/Polar Compounds and HDAC Inhibitors. Hybrid/polar compounds were named on the basis of their physicochemical properties to have polar groups at their terminals and a highly hydrophobic linker in the middle of the molecule.[20] **1a** (Figure 3A),[21] the most promising agent of hybrid/polar compounds, reached phase II clinical trials on MDS and AML.[22] The discouraging results of this trial (high doses, suboptimal activity, and important toxicities) urged researchers to further optimize the potency of **1a**. Since tri- and tetraamides proved to be ineffective,[23] replacement of the acetamide moiety with the metal chelating *N*-hydroxyamide emerged as an optimal one, and as a result, the synthesized suberic bishydroxamic acid (SBHA) displayed significantly increased differentiation-induced potency to leukemic cells compared to **1a**.[24] Structure variations led to the discovery of **1b**, later named as vorinostat (Figure 3A), that was the most potent compound at that time.[25] **1b** proved to be a HDACI[26] and after extensive clinical investigation was launched recently as an FDA approved drug for the treatment of the rare cutaneous T-cell lymphoma.[20] Also, romidepsin very recently (November 2009) gained FDA approval for the same pathological condition.[27]

Since HDACIs have been extensively reviewed recently,[28] attention will be given here on HDACIs that are in clinical trials for leukemia. HDACIs are studied either alone or commonly in combination with DNMT inhibitors. JNJ16241199[28] is in preclinical development for leukemias, and pyroxamide and SB939[28] are in phase I clinical trials for hematological malignancies. Further HDACIs in development are belinostat, entinostat, mocetinostat, romidepsin, panobinostat, and **1b**, which have been advanced to phase III clinical trials as a monotherapy or in combination with other agents (more information about these compounds can be found in Table 2). Combination trials of these inhibitors with hypomethylating agents are also commented in the paragraphs below.

5.2. DNMT Inhibitors. DNMTs are the enzymes responsible for DNA methylation in mammals, being part of the epigenetic regulation of gene expression in eukaryotic cells. Epigenetic regulation of gene expression is the "switch" turning genes on and off by DNA methylation of CpG islands and/or histone modifications (acetylation, methylation) in specific lysine and arginine residues.[28−31] Inactivation of tumor suppressor genes is a phenomenon often observed in the initiation and progression of leukemia as well as in malignancy in general. In a recent study,[30] the genes *THBS1* and *Ril* were found to be 100% hypermethylated in leukemia cell lines, and *ECAD*, *P15*, *ER*, *TMS1*, and *THBS4* were over 60% hypermethylated in leukemia cell lines. Furthermore, in a study in which bone

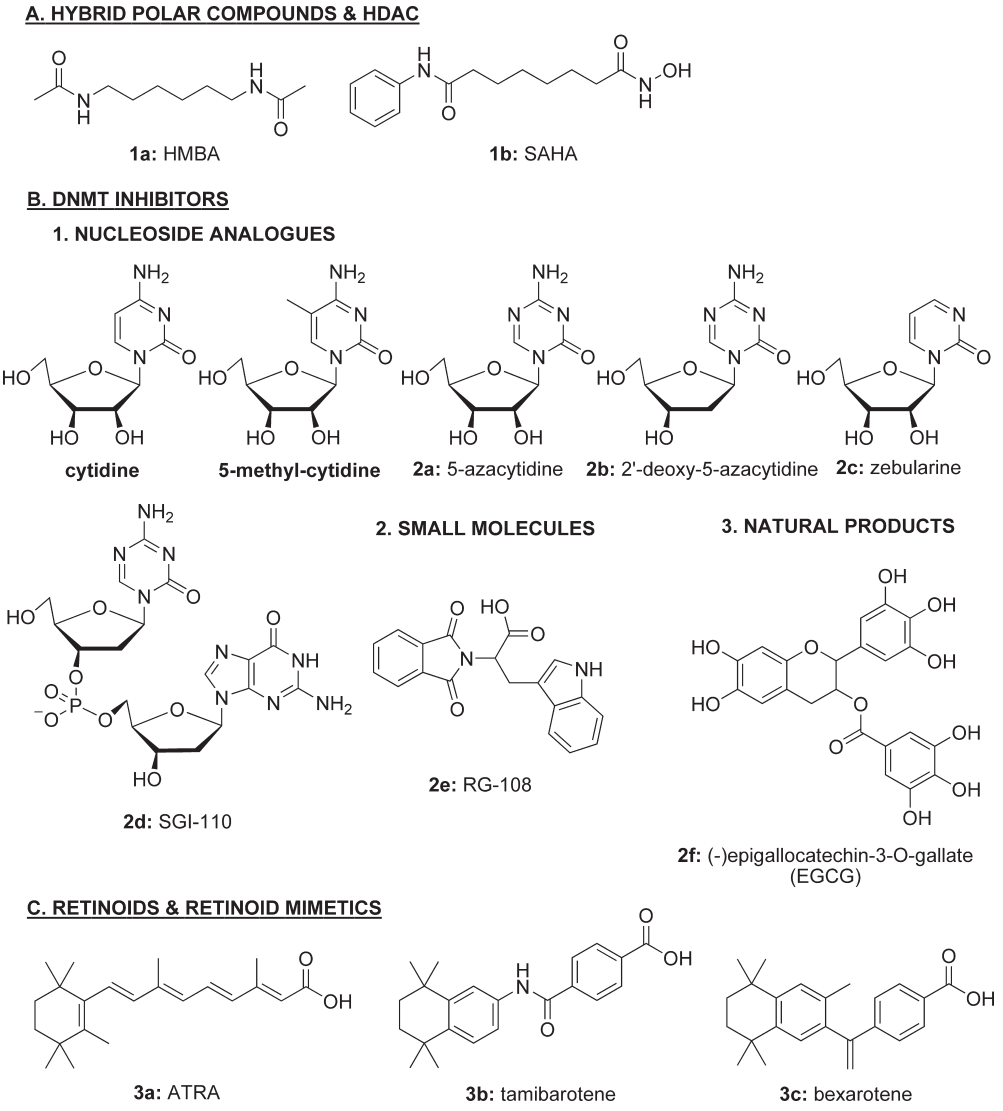

Figure 3. Differentiation inducing agents (DIA).

marrow samples from AML patients were analyzed, it was found that 95% had at least one of the following genes hypermethylated and 75% had two or more. The genes referred to are calcitonin, estrogen receptor, *E*-cadherin, *p15*, *p16*, *Rb*, *GST-Pi*, and *HIC1*.[29] Also, the role of DNA hypermethylation in acute lymphocytic leukemia (ALL) initiation and progression was presented recently.[32] On this basis, hypomethylating agents could prove to be useful tools in leukemia therapy and some of them are in clinical trials or in clinical use.

Hypomethylating agents, or more specifically DNMT inhibitors, can be divided into two categories[33] with respect to their chemical structure: nucleoside analogues and non-nucleoside inhibitors. The second can be further divided into small molecule inhibitors (SMIs) and natural products.

The most studied nucleoside analogues are **2a**, **2b**, and zebularine[34] (**2c**) (Figure 3B1). The first two are highly cytotoxic compounds, but at lower concentrations they can induce differentiation of leukemic cells and have an impact on DNA methylation.[16,17,35] So far, they are FDA approved for the treatment of MDS and chronic myelomonocytic leukemia (CMML), but they also are included in a number of clinical trials, alone or in combination, for other malignancies (Tables 3 and 4).

2b is incorporated in the DNA where it traps DNMTs. DNMTs methylate cytosine residues at the 5′-C. Since the 5′-C is replaced with a nitrogen atom in the molecule of **2b**, no methylation can take place and a covalent bond is generated between the enzyme and the triazine nucleosides after its incorporation into DNA.[36] In low doses the result is hypomethylation, but in higher doses steric inhibition of DNA replication and repair due to the bulky adducts is thought to be the cause of **2b** cytotoxicity.[37] **2a** is thought to be converted to **2b** by ribonucleotide reductase before its incorporation into DNA. Besides, its cell growth inhibitory effect is also caused by its incorporation into RNA molecules and by affecting protein synthesis.[36] **2c**, initially synthesized as a cytidine deaminase inhibitor,[34] is a deaminocytosine nucleotide that was developed to overcome **2a** and **2b** instability in neutral and acidic aqueous solutions in order to make oral administration of the drug feasible.[38] However, low clinical efficiency, mostly due to cytidine deaminase inactivation and antagonism with the increased cytidine and deoxycytidine levels, has hindered its further development.[38] Finally, a second generation inhibitor currently under preclinical development is the **2b** dinucleotide SGI-110[39] (**2d**, Figure 3B1) that has proved advantageous in the manner of cytidine

Table 2. Significant HDAC Inhibitors in Clinical Trials for Leukemia[a]

compd	category	leukemia clinical trials	phase (NCT ID)[b]
belinostat (PXD101, NSC 726630), TopoTarget	hydroxamic acid	AML	phase II (NCT00357032)
		AML, combination with idarubicin	phase I/II (NCT00878722)
entinostat (MS-275, MS275, SNDX-275, NSC 706995), Syndax Pharmaceuticals	benzamide	MDS, AML, ALL, combination with GM-CSF	phase II (NCT00462605)
		hematologic cancer	phase I (NCT00015925)
mocetinostat (MGCD0103), MethylGene	benzamide	CLL	phase II (NCT00431873)
		untreated AML, high risk MDS	phase II (NCT00374296)
		high risk MDS, AML	phase I/II (NCT00324220)
		MDS, leukemia	phase I (NCT00324194, NCT00324129)
pivanex, Titan Pharmaceuticals	short chain fatty acid	CLL	phase II (NCT00083473)
pyroxamide	hydroxamic acid	advanced cancer	phase I (NCT00042900)
SB939, SBIO	hydroxamic acid	solid tumors, hematologic malignancies	phase I (NCT00741234)
romidepsin (FR901228, FK228, NSC630176), Gloucester Pharmaceuticals	bicyclic depsipeptide	AML	phase II (NCT00062075)
		MDS, AML, NHL	phase II (NCT00042822)
		CLL and small lympholytic lymphoma, combination with bortezomib	phase I (NCT00963274)
		hematologic cancer	phase I (NCT00024180)
panobinostat (LBH 589, LBH589, NVP-LBH589, LBH489B, LBH489A), Novartis	hydroxamic acid (iv and po) flexibility in developing combination regiments	AML	phase II (NCT00880269)
		CML	phase II/III (NCT00449761, NCT00451035)
		ALL, AML	phase II (NCT00723203)
		AML, combination with idarubicin	phase I/II (NCT00840346)
		CML, combination with imatinib	phase I (NCT00686218)
		T-cell lymphoma and leukemia	phase II (NCT00699296)
vorinostat (zolinza, SAHA, **1b**), Merck	hydroxamic acid (FDA approved Oct 2006 for cTCL)	AML	phase II (NCT00305773)
		AML, MDS, combination with sorafenib	phase I (NCT00875745)
		leukemia, MDS, combination with idarubicin	phase I (NCT00331513)
		AML, MDS, combination with idarubicin and cytarabine	phase II (NCT00656617)
		AML, combination with gemtuzumab and azacytidine	phase I/II (NCT00895934)
		acute leukemia, MDS, combination with cytarabine and etoposide	phase I (NCT00357305)
		AML, combination with gemtuzumab	phase II (NCT00673153)
		mantle cell lymphoma, CLL, NHL, combination with cladribine and rituximab	phase I/II (NCT00764517)
		B-cell CLL and small lymphocytic lymphoma, combination with rituximab and fludarabine phosphate	phase II (NCT00918723)
		acute leukemia or CML, combination with flavopiridol	phase I (NCT00278330)
		AML, MDS, combination with bortezomib	phase II (NCT00818649)
		CML, ALL, combination with dasatinib	phase I (NCT00816283)
		ALL, lymphoblastic lymphoma, with decitabine and combination chemotherapy	phase I (NCT00882206)

[a] Source: http://www.clinicaltrials.gov/. Abbreviations: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; cTCL, cutaneous T-cell lymphoma; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma. [b] Represents the National Clinical Trials identifier (NCT ID).

Table 3. Azacytidine and Decitabine in Clinical Trials for Leukemia[a]

compd	conditions	phase (NCT ID)[b]
azacytidine (**2a**) (5-azacytidine, NSC-102816, Vidaza), Celgene	CLL	phase II (NCT00413478)
	AML	phase I/II (NCT01016600) phase II (NCT00728520, NCT00387647, NCT00739388)
	CML	phase II (NCT00813124)
	AML, MDS	phase II (NCT00795548, NCT00915785)
		phase III (NCT00887068, NCT00422890)
	AML, CMML, MDS	phase I (NCT00528983, NCT00761722)
	AML, MDS, combination with VPA and ATRA	phase I/II (NCT00326170)
		phase II (NCT00339196)
	AML, combination with bortezomib	phase I (NCT00624936)
	AML, combination with standard chemotherapy	phase II (NCT00915252)
	AML, combination with gemtuzumab	phase I/II (NCT00766116)
		phase II (NCT00658814)
	AML, combination with lenalinomide	phase I/II (NCT00890929)
	AML, MDS, combination with Ara-C	phase I/II (NCT00569010)
	AML, MDS, combination with lenalinomide	phase I (NCT00923234)
	MDS, CMML, combination with AsO_3	phase II (NCT00118196)
decitabine (**2b**) (2′-deoxy-5-azacytidine, dezocytidine, NSC-127716, Dacogen), Eisai	AML	phase III (NCT00260832)
		phase II/III (NCT00398983)
		phase II, (NCT00358644, NCT00492401)
		phase I (NCT00538876, NCT00986804, NCT00882206)
	ALL	phase I (NCT00349596)
	AML, combination with bexarotene	phase I, NCT01001143
	CML	phase II (NCT00042003, NCT00042016, NCT00041990)
	CMML	phase II (NCT00113321)
	AML, MDS	phase II (NCT00760084)
		phase I (NCT00049582)
	AML, ALL	phase I (NCT00042796)
	AML, combination with AsO_3 and ascorbic acid	phase I (NCT00671697)
	AML, combination with rapamycin	phase I (NCT00861874)
	AML, combination with VPA + ATRA	phase II (NCT00867672)
	AML, combination with bortezomib	phase I (NCT00703300)
	CML, combination with imatinib	phase II (NCT00054431)
	AML, MDS, combination with gemtuzumab	phase II (NCT00882102)
	AML, MDS, combination with clofarabine	phase II (NCT00778375)
	AML, MDS, combination with G-CSF and Ara-C	phase II (NCT00740181)
	leukemia, MDS, CML	phase I/II (NCT00002832, NCT00002831)
	leukemia, myelodysplastic syndromes, myeloproliferative disorders, combination with romidepsin	phase I (NCT00114257)

[a] Source: http://www.clinicaltrials.gov/. Abbreviations: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; ATRA, all trans retinoic acid; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; VPA, valproic acid. [b] Represents the National Clinical Trials identifier (NCT ID).

deaminase inactivation but has no improved stability in comparison to **2b**.[40]

In the field of SMIs, a number of compounds have been found to possess DNMT's inhibitory activity. At first, the approach to test known drugs for inhibitory activity was used, and hydralazine, procaine, and procainamide were recognized as DNMT inhibitors.[41] A series of constraint analogues of procaine were also synthesized but with no inhibitory activity.[42] Also, a second approach applied was the rational design by using an in silico method that resulted in the identification of RG-108[43] (**2e**) (Figure 3B2) as a DNMT1 inhibitor. **2e** was found to inhibit DNMT1 without binding covalently to it and caused DNA hypomethylation and reactivation of tumor suppressor genes,[44] a fact of therapeutic significance in cancer to restrict cell proliferation.

The most intriguing natural products that exhibit DNMT inhibitory activity are (−)-epigallocatechin 3-gallate (EGCG, **2f**, Figure 3B3)[45] and curcumin,[46] though the multiple biological effects of these compounds and their antioxidant nature may intervene and deteriorate the evaluation of their beneficial anticancer activity.

So far, the most promising results come from the field of nucleoside analogues, since **2a** and **2b** induce strong demethylation as well as reactivation of tumor suppressor genes with the drawback of cytotoxicity and pH-related instability. **2c** and **2e** cause mediocre demethylation with mild or no cytotoxicity and can be used as lead compounds for further development.[47]

5.3. Combining HDAC and DNMT Inhibitors. As mentioned before, gene promoter hypermethylation and core histone hypoacetylation are epigenetic modifications that

Table 4. Clinical Trials Combining HDAC and DNMT Inhibitors[a]

HDAC and DNMT inhibitors	conditions	phase (NCT ID)[b]
VPA + azacytidine	AML, MDS	phase II (NCT00382590)
sodium phenylbutyrate + azacytidine	AML, MDS	phase I (NCT00004871)
	AML, NHL, nSCLC, multiple myeloma, prostate cancer	phase II (NCT00006019)
VPA + decitabine	AML, MDS	phase I/II (NCT00075010)
		phase II (NCT00414310)
	AML, CLL, small lymphocytic lymphoma	phase I (NCT00079378)
belinostat + azacytidine	advanced hematological malignancies	phase I (NCT00351975)
entinostat + azacytidine	CML, AML, MDS	phase I (NCT00101179)
		phase II (NCT00313586)
mocetinostat + azacytidine	AML	phase II (NCT00666497)
panobinostat + azacytidine	MDS, CMML, AML	phase I (NCT00946647)
panobinostat + decitabine	MDS, AML	phase I/II (NCT00691938)
romidepsin + decitabine	leukemia, MDS	phase I (NCT00114257)
vorinostat + azacytidine	AML, MDS	phase II (NCT00948064)
		phase I/II (NCT00392353)
vorinostat + decitabine	NHL, AML, ALL, CML	phase I (NCT00275080, NCT00479232)
	hematologic cancer	phase I (NCT00357708)

[a] Source: http://www.clinicaltrials.gov/. Abbreviations: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; nSCLC, non-small-cell lung cancer; VPA, valproic acid. [b] Represents the National Clinical Trials identifier (NCT ID).

cause silencing of tumor suppressor genes and contribute to malignancy. A connection between hypermethylated gene promoter regions and histone modifications is emerging in the last couple decades, leading to thorough examination of potential synergistic anticancer activity of these two separate mechanisms of epigenetic regulation.[48−50] The finding that the HDACI trichostatin (TSA) alone cannot up-regulate the expression of hypermethylated genes but that this becomes feasible upon co-treatment with the DNMT inhibitor **2b**[51] generated the idea of combining the two agents in therapeutics in order to act synergistically for the re-expression of genes. Thus, sequential administration of a DNMT inhibitor followed by a HDACI could potentially increase the response rate and duration to benefit the therapeutic outcome. Alternatively, one also can consider the possibility of combining DNMT inhibitors with agents causing histone lysine demethylation, as recently proposed.[52]

The numerous clinical trials that employed DNMT and HDACI are described in Table 4. The initial studies with HDACI such as valproic acid (VPA) and phenyl butyrate were insufficient to prove a distinct synergism, but this could be attributed to their low HDACI activity.[36] In order to further explore the therapeutic potential of such an approach, more potent small molecule HDACIs should be evaluated in newly designed clinical trials. Some of them are now in progress.

5.4. Retinoids and Retinoid Mimetics. Targeting the nuclear retinoid receptors has so far been proven as a reliable and effective method in the differentiation therapy of APL. Since the initial FDA approval of all-trans retinoic acid (ATRA, **3a**, Figure 3C),[53] extensive studies have been taking place in order to reduce its severe side effects, improve its stability, and overcome the acquired tumor resistance. Modifications made on **3a** include the addition of heteroatoms to the candidate molecules and the replacement of the polyene chain with an aromatic ring, which resulted in the synthesis of the retinoid acid receptor α (RAR_α) selective tamibarotene (AM80, **3b**, Figure 3C) (EC_{50} = 45, 235, and 591 nM, for RAR_α, RAR_β, and RAR_γ, respectively).[54] **3b** exhibits greater stability and bioavailability than **3a** because of the absence of the polyene chain and its less hydrophobic character. Also, it appears to bind poorly to cellular retinoic acid-binding proteins (CRABPs). As a result, **3b** has the potential to overcome ATRA-acquired resistance, since it is related to CRABPs that enhance **3a** catabolism and result in inefficiency.[54,55] Furthermore, lack of binding to RAR_γ receptor seems to diminish adverse effects, a fact that also favors **3b** compared to **3a**.[56] **3b** has also proved to be effective in patients that relapsed after complete remission with **3a** (58% against 20% complete remission with second treatment with **3a**). Further clinical trials enhanced these clinical results to the point of **3b** commercial availability (2005 in Japan, Toko Pharmaceuticals) and also fast drug designation for relapsed or refractory APL by the FDA (June 2007).

As an alternative route to overcome the above-mentioned **3a**'s drawbacks in adverse effects and emergence of resistance, targeting of the retinoid X nuclear receptor (RXR) emerged. Ligands that bind selectively to RXR emerged, and the term "rexinoids" arose.[55] Literature of rexinoids accumulated,[57−63] but the most important compound of this category is considered to be the FDA approved bexarotene (**3c**, Figure 3C)[64] for the treatment of cutaneous T-cell lymphoma. The putative use of **3c** on leukemia is still in preclinical stages, and for that reason it will not be extensively reviewed in this paper. Regardless of their multifunctionality, none of these retinoid compounds have entered clinical trials for the treatment of leukemia, so an extensive analysis is also out of the scope of this Perspective.

6. Induction of Apoptosis in Leukemias

6.1. Bcl-2 Inhibitors. Extensive therapeutic exploration of apoptotic pathways has established B-cell lymphoma (Bcl) proteins as a new target for cancer therapy over the past years. Antisense RNA technology was the first approach applied to suppress Bcl-2 function, but subsequently the development of SMIs was considered as a more promising therapeutic strategy. The target region of the Bcl-2 protein was identified within a hydrophobic groove, the so-called BH3 binding region. The hunt to "get into the groove"[65] started with the screening of natural product libraries and continued with computer-based screening methodology of small molecules and the application of NMR fragment screening. Up to today, several candidates have been in preclinical development with very promising results, and some of them even reached clinical trials as monotherapy

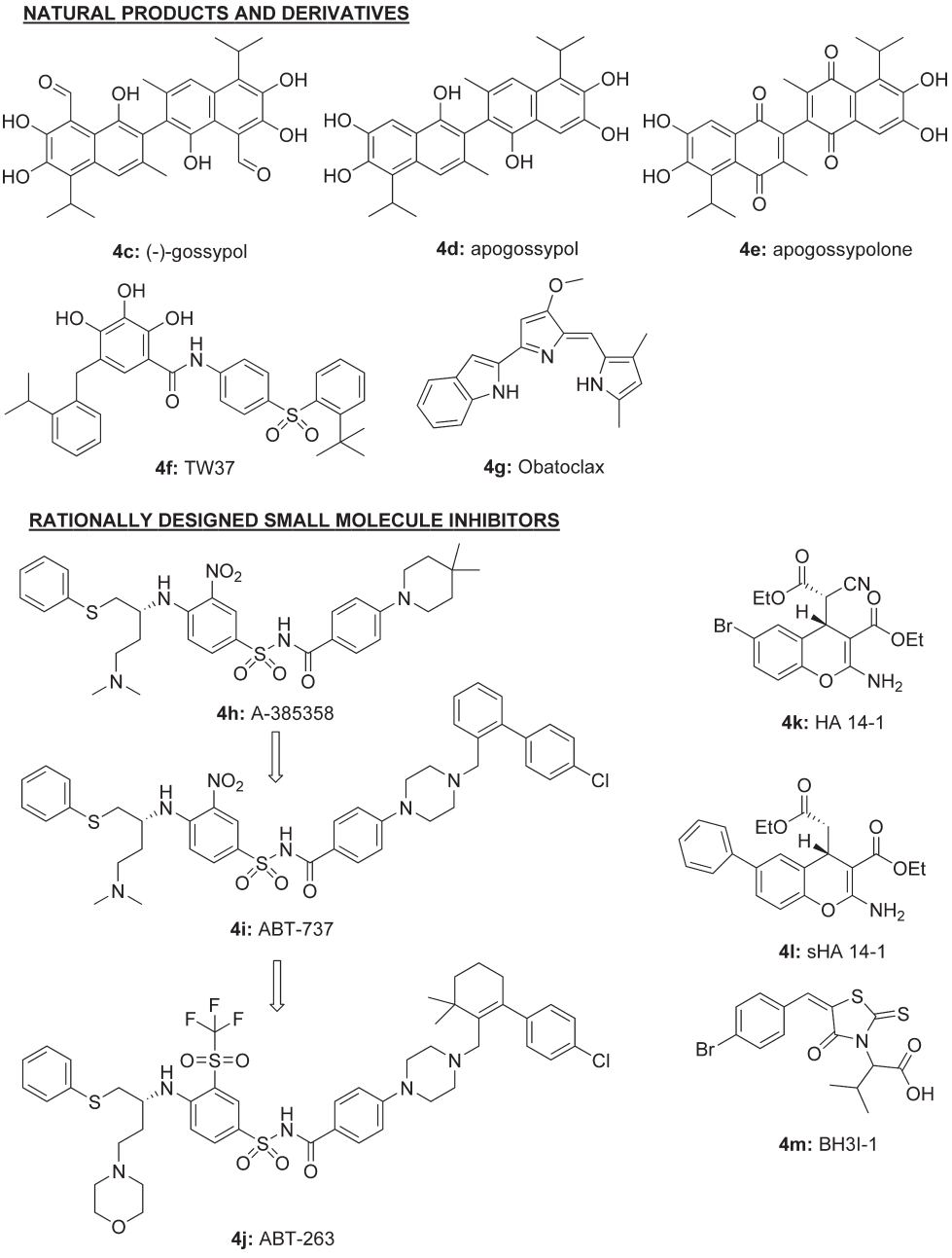

Figure 4. BH_3 mimetics (BCL binding molecules).

or combination therapy for leukemia. Most clinical trials are directed toward chronic lymphoid leukemia (CLL), since overexpression of Bcl-2 is observed in this type of hematologic malignancy.[66]

Although focus will be imposed on the development of SMIs regarding the *BCL-2* gene, two of the most important paradigms of antisense technology with successful introduction into clinical practice must be mentioned: oblimersen sodium[67] (Genta, G3139, **4a**) and SPC 2996[68] (**4b**). In particular, **4a** is a 18-nucleotide antisense molecule targeting the initiation codon region (codons 1−6) of BCL-2 mRNA.[67] Oblimersen sodium has successfully completed a phase I/II clinical trial on chronic myeloid leukemia (CML) treatment as a monotherapy and is currently under further clinical investigation (up to phase III trials) as combination therapy for CLL, CML, AML, and ALL.[14] Some of the biodistribution problems that arose because of its polyanionic character and rapid metabolism are hoped to be solved through PEGylation of the molecule.[69] Furthermore, applying the new technology of locked nucleic acids, **4b** is a 16-nucleotide antisense molecule that has reached the clinic and is now examined in a phase I/II trial in patients with relapsed or refractory CLL.[68]

Screening of natural products for potential Bcl-2 inhibitors revealed that black tea polyphenols, and especially **2f**, inhibit Bcl-2 function, although with limited specificity, since these molecules also affect other pathways related to cell survival.[70] However, a plethora of natural products has already been studied for *BCL-2* gene suppression including antimycin A,[71] chelerythrine,[72] and purpurogallin,[73] but the most intensively studied and currently in clinical trials is (−)-gossypol [AT-101 (**4c**) Figure 4, Table 5].

4c is currently under phase II clinical trials for CLL. **4c** binds Bcl-2 (K_i = 320 nM), Bcl-X_L (K_i = 480 nM), and Mcl-1 (K_i = 180 nM).[74] In studies where cell population

Table 5. BH_3 Mimetics in Clinical Trials for Leukemia[a]

compd	target	trials on leukemia	further clinical trials
obatoclax mesylate (**4e**) (GX15-070), Gemin X Biotechnologies	Bcl-2, Bcl-XL, Bcl-w, Mcl-1	CLL (phase I/II)	MDS (phase II), myelofibrosis with myeloid metaplasia (phase II), mantle cell lymphoma (phase I), nSCLC (phase I/II), Hodgkin's disease (phase II), NHL (phase I/II), SCLC (phase I/II), MM (phase I/II)
ABT-263 (**4j**) (RG7423), Abbott Laboratories	Bcl-XL, Bcl-2, Bcl-w, Bcl-B	AML (phase II) hematologic malignancies (phase I/IIa)	SCLC (phase I), lymphoma, CD20(+) (phase I), solid tumors (and combination) (phase I)
(−)-gossypol (**4a**) (AT-101, levo-gossypol), Ascenta Therapeutics	Bcl-2, Bcl-XL, Bcl-w, Mcl-1	CLL (B-cell) (phase I), phase II B-cell malignancies (CLL) phase II, combination with lenalidomide (CLL) phase I/II, combination with rituximab (CLL) phase II	prostate cancer (phase II), lung cancer (phase I/II), adrenocortical carcinoma (phase II), brain and CNS tumors (phase I/II), lymphoma (phase I), nSCLC (phase I), esophageal or GE junction cancer (phase I/II), SCLC (phase I/II), follicular lymphoma (phase II)

[a] Source: Oncology KnowledgeBase http://oncologyknowledgebase.com/Login.aspx?ReturnUrl=%2fDefault.aspx. Abbreviations: AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; CNS, central nervous system; GE, gastroesophageal; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma; nSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer.

isolated from patients with CLL was used, **4c** exhibits an EC_{50} of 2 μM.[75] But despite its potent binding and good pharmacokinetic profile, this molecule remains a highly reactive aldehyde and thus exhibits important cellular toxicity. On this basis and with the prospect to improve Bcl binding, several analogues have emerged: apogossypol (**4d**), apogossypolone (**4e**), and TW37 (**4f**) (Figure 4). The first two, which are in preclinical development, do not possess the aldehyde group of gossypol and appear to better target Bcl-2 and Mcl-1.[76,77] The synthetic **4f** molecule appears to be the most promising with submicromolar IC_{50} values for Bcl-2 and Mcl-1 and micromolar values for Bcl-X_L.[78] Its binding affinity to Mcl-1 is of extreme importance to avoid the implication of drug resistance,[79] and the fact that **4f** is a pan-Bcl inhibitor establishes it as a promising compound for further development.

Furthermore, obatoclax mesylate (**4g**, Gemin X Biotechnologies, Figure 4) derived from lead optimization of prodigiosin, tripyrrole natural products[80] has reached phase II clinical trials for the treatment of CLL and AML. With an EC_{50} of 1.7 μM exhibited in B-cells derived from CLL patients,[81] **4g** binding to Bcl-2, Bcl-w, Bcl-X_L, and Mcl-1 is in the low micromolar range, although there is certain variability in the values reported, probably due to its poor solubility.[82] However, a report from a recent phase I trial has confirmed a very short $t_{1/2}$ and modest single agent activity of **4g**, thus suggesting a possible use in combination therapy only.[83]

Finally, in the area of rational design, additional SMIs have been identified by computational screening. These are HA14-1[84] (**4k**), its soluble analogue sHA14-1[85] (**4l**), and BH3I-1 (**4m**) and analogues.[86] Such molecules are some of the Bcl-2 suppressors identified thus far, but none of them advanced to clinical development. Abbott Laboratories through NMR fragment screening developed a series of compounds including A-385358[87] (**4h**) and ABT-737[88] (**4i**). Such experience led researchers recently to identify ABT-263 (**4j**), which exhibits acceptable oral bioavailability and is now under clinical development.[89] As far as these molecules are concerned, the Bcl-X_L hydrophobic groove was initially divided into two smaller sites that were separately targeted by SMIs, which were linked and slightly optimized to constitute a single precursor molecule bearing the acylsulfonamide moiety (Bcl-X_L K_i = 0.036 μM).[90] Attempts to minimize binding to human serum albumin yielded the molecule **4h**.[87] At this point, Bcl-X_L was specifically targeted, so **4h** bound much less tightly to Bcl-2 (K_i = 67 nM), compared with the binding to Bcl-X_L (K_i = 0.8 nM). In order to achieve dual inhibition of both Bcl-2 and Bcl-X_L, binding deeper in the groove was imperative and the piperidine moiety was under the scope at this time. Replacement with a piperazine moiety that is further substituted with a 4′-chlorobiphenyl moiety was the most favorable, and **4i** was discovered.[88] **4i** had high affinity for both Bcl-2 and Bcl-X_L (8 and 30 nM, respectively), and CLL cells proved to undergo apoptosis at very low concentrations (EC_{50} = 4.5 nM and EC_{50} = 7 nM),[91,92] but this compound stayed at the preclinical level because of low aqueous solubility (< 1 μg/mL) and poor oral bioavailability.[93] Three sites were then selected as the most prone to further optimization: (1) the dimethylamino group was converted to morpholine in an effort to improve the metabolic properties of the molecule, though with the drawback of reduced potency; (2) the nitro group, a site of potent toxicity, was replaced with a trifluoromethylsulfonyl group (in order to retain the electron-withdrawing properties and as a consequence the low pK_a of the sulfonamide group required for activity); (3) the biphenyl moiety transformed one phenyl ring to an appropriate cycloalkene in order to conserve rigidity but excluded another metabolically active site.[89] The result was the generation of **4j** that, despite its poor water solubility, was up to 50% bioavailable in animal models after oral delivery[94] because of its limited metabolism. **4j** attained high affinity for Bcl-2, Bcl-X_L, and Bcl-w (< 1 nM), but no activity against Mcl-1 was observed. In ALL xenograft models, **4j** induced complete tumor regression[94] and it advanced to clinical trials for hematologic malignancies.

6.2. Tyrosine Kinase Inhibitors. Protein kinases are one of the most popular targets in the developmental therapeutics area of oncology. Since kinases mediate signaling pathways related to tumor suppressor genes or oncogenes and are implicated in the regulation of apoptosis, proliferation, invasion, and differentiation, targeting them has been proved to be an efficient pharmacological intervention in malignancy. Since tyrosine kinase targeting in hematological malignancies has been recently reviewed by Chase and Cross,[95] attention here will be only to the inhibitors, with emphasis given on their structure−activity

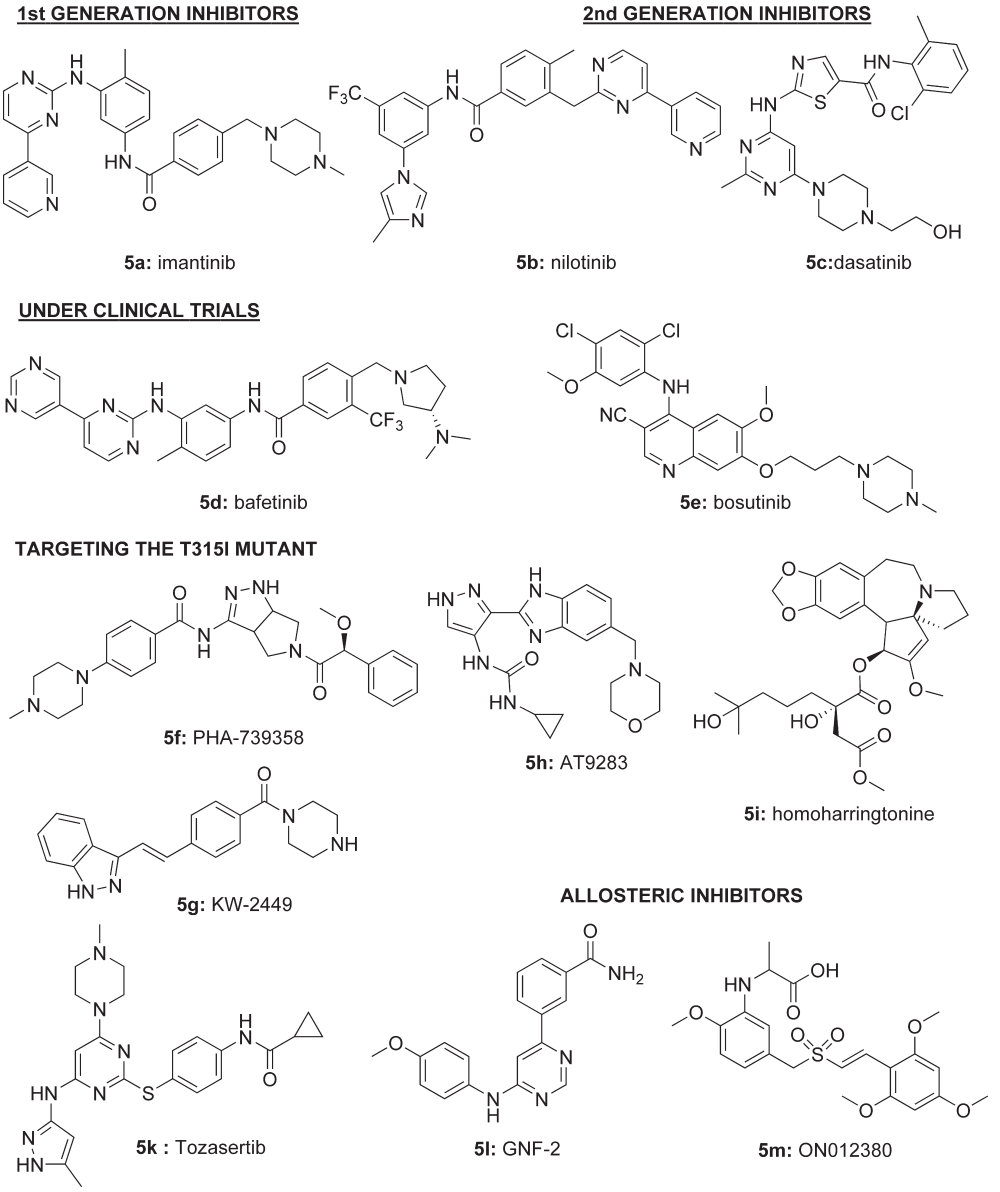

Figure 5. Bcr-Abl tyrosine kinase inhibitors.

relationships and kinase inhibitory profile. Following imatinib's (**5a**) breakthrough few years ago, seven kinase inhibitors were developed and entered the market, namely, **5a** [launched in 2001, Novartis, for CML, gastrointestinal stromal tumors (GIST), and hypereosinophilic syndrome (HES)], erlotinib [2005, OSI Pharmaceuticals/Gentech/Roche, for non-small-cell lung cancer (NSCLC) and pancreas cancer], gefitinib (2005, Astra-Zeneca, for NSCLC), dasatinib (**5c**) (2006, Bristol-Myers Squibb, for imatinib resistant CML), nilotinib (**5b**) (2007, Novartis, for imatinib resistant CML), sorafenib (**6f**) (2007, Bayer/Onyx, for renal cancer), sunitinib (**6d**) (2007, Pfizer, for renal cancer and imatinib refractory GIST), and lapatinib (2007, GlaxoSmithKline, for breast cancer). Since additional small molecule kinase inhibitors are under clinical and preclinical development for treatment of various malignancies other than leukemia, their coverage is beyond the scope of this paper.

6.2.1. Bcr-Abl Kinase Inhibitors. Targeting the chimaeric Bcr-Abl tyrosine kinase found in the majority of Ph^+ (reciprocal translocation between chromosomes 9 and 22) CML patients, **5a** was developed as an effective inhibitor from a series of protein kinase C (PKC) inhibitors where the phenylaminopyrimidine core proved to have leadlike properties.[96] Structure optimization led to the phenylaminopyrimidine compound **5a**[8,97] (Figure 5) that had the appropriate solubility and bioavailability to proceed to the clinic.[98] **5a** proved to be a very selective inhibitor among various kinases (Abl, c-Kit, Lck, PDGFR, and CSF-1R),[99] a property believed to result from its binding to the inactive conformation of Bcr-Abl, the so-called "DGF-out" conformation.[100] But while selectivity is generally observed, the vulnerability to mutations of this class of inhibitors is the common cause of therapeutic inefficiency. Specifically, the "gatekeeper" (T315I) and also other mutations of Bcr-Abl turn the balance toward the active conformation,[101] rendering **5a** and other inhibitors inactive.

This is partially attributed to the occurrence of specific mutations in the gene encoding the Bcr-Abl fusion tyrosine kinase that cause conformational changes and alter **5a**'s affinity and effectiveness. Another Bcr-Abl independent mechanism of imatinib resistance is overexpression of the efflux protein glycoprotein (P-gp) in CML that it is likely to reduce the intracellular concentrations of **5a** to subtherapeutic levels, since **5a** is a Pg-p substrate.[102] To this end, the

accumulated knowledge thus far from structural and mechanistic insights of the polymorphic imatinib-insensitive Bcr-Abl kinase were further exploited to override the emergence of **5a** clinical resistance in the newer protein kinase inhibitor (PKI) drugs.[7,103] Indeed, such a direction has led to the clinical development of antileukemia multitarget kinase inhibitors **5b** and **5c** (Figure 5) that are potent inhibitors of multiple kinases, inhibit both wild-type and mutated Bcr-Abl kinase, and are approved for the treatment of imatinib-resistant CML patients.[7] Unlike **5a**, **5c** is not a substrate of P-gp and also human organic cation transporter 1 (hOCT1), properties that are also beneficial in treating imatinib-resistant CML.[102,104]

5b, a phenylaminopyrimidine like **5a**, was developed as a less stringent **5a** analogue in order to bind putative mutated Bcr-Abl domains.[105] **5b** is 20-fold more potent than **5a**, has increased selectivity, and was found to retain activity against 14 of 15 imatinib-resistant Bcr-Abl mutants.[106] **5c**, on the other hand, an aminothiazole Src/Abl kinase inhibitor[107,108] with activity on other kinases (Kit, PDGFR, ephrin A receptor kinase,[109] and the Tec kinase Btk[110]), has even greater affinity (~325-fold more potent than **5a**) due, at least partially, to its binding in inactive and active conformations of Bcr-Abl kinase.[111] In CML cell models bearing imatinib-resistant mutants, **5c** was found to be active in 14 of 15 cases.[112] Recently, ^{18}F-dasatinib was synthesized to be used as a prognostic tool for kinase inhibitory activity with PET technology.[113] Also in clinical development are the benzamide Abl/Lyn kinase inhibitor bafetinib[114] (**5d**, Figure 5) and the 3-quinolinecarbonitrile Src/Abl inhibitor bosutinib[115,116] (**5e**, Figure 5). **5e** is currently in phase III clinical trials for CML, whereas **5d** is in phase I. All these compounds were proved to be active against the majority of imatinib-resistant CML cells. The exception in all cases is the gatekeeper mutation T315I of Bcr-Abl, and resistance is thought to be caused by the loss of a hydrogen bond within the side chain of this residue.[117]

The binding of chemical molecules to kinases has been extensively studied, along with the selectivity factors affecting it and their modes of binding. Crystallographic studies complemented with molecular simulation approaches have provided the positions for inhibitor/enzyme interactions for each inhibitor.[118] A common structural characteristic of most of the inhibitors synthesized is the pyrimidine core, also present in ATP. Each inhibitor binds through different sites in the ATP binding cleft of Bcr-Abl, and apart from the structural flexibility of inhibitors, also of great importance in binding to mutant Bcr-Abl forms is the size/volume of the gatekeeper's side chain because it allows access to the hydrophobic cleft located behind it.[119] Loss of activity through disruption of hydrogen bond formation with the side chain of the gatekeeper residue is another resistance mechanism, as mentioned above. In order to override this "Achilles' heel" in CML therapy, many compounds are currently in preclinical and clinical development.

Multitargeted kinase inhibitors may afford a solution due to "off-target" activities they exhibit.[7] XL228[120] and PHA-739358[120] (**5f**), KW2449[121] (**5g**), and AT9283[122] (**5h**) (identified as Aurora kinase inhibitors, Figure 5) have potency against the mutant T315I and are in clinical development for CML therapy.[112] The aforementioned inhibitors are ATP-competitive but share the property of no interaction with the gatekeeper residue, rendering them capable of T315I inhibition, and this could be clinically effective in resistant CML patients.[123] Of novel mechanism, but not yet fully characterized, is homoarringtonine[124] (**5i**, Figure 5), a proapoptotic cephalotaxine ester, in phase II clinical trials for patients with T315I mutant CML. Along with **5f**, DCC-2036[117] (**5j**, structure not disclosed) is also under clinical development. **5j** is an inhibitor targeting "switch pockets" distant from the gatekeeper region. However, tozasertib (**5k**, Figure 5), a multitargeted Aurora kinase inhibitor, was recently withdrawn from a phase II clinical trial because of the rise of significant cardiotoxicity (see Table 6 for information on second generation inhibitors).

Another approach to circumvent resistance is the use of "allosteric" inhibitors that are non-ATP-competitive. Of these molecules, attention has been drawn to GNF-2[125] (**5l**, Figure 5), a compound that binds to the myristate binding cleft at the N-terminus, resulting in stabilization of the protein in its inactive state, and ON012380[126] (**5m**, Figure 5), a potent inhibitor that blocks the substrate-binding site of Bcr-Abl. Unfortunately, these molecules have not been registered for clinical evaluation so far. Finally, other strategies under development to circumvent the emergence of resistance in CML patients are the application of innovative molecules such as small interfering RNAs (siRNAs), transcript-specific ribozymes, antisense oligonucleotide and peptide nucleic acids.[127]

Even though selectivity was initially sought in order for an inhibitor to proceed to the clinic, recently focus has been on multitargeted kinase inhibitors. With multitargeted kinase inhibitors, by the use of a single inhibitor, multiple components of the same signaling pathway can be regulated or multiple signaling pathways and cellular processes can be related to cell survival, differentiation, or apoptosis, thus leading to a more efficacious therapy and eradication of resistant cells. In such a case, more than one leukemia type (e.g., AML, ALL) or even solid tumors could be eventually treated. On the other hand, selectivity is sought in order to minimize severe side effects of a broad range kinase inhibitors. Additional studies on novel and current inhibitors may provide a solution to this dilemma to keep in balance the specificity with the multitargeted capacity, in terms of improved therapeutic outcome. Alternative strategies to address this issue discussed above should also provide ground for advancement in drug development in this area, in order for us to lead to safer therapy with diminished resistance and remissions.

6.2.2. FLT3 Inhibitors. Activating mutations of the fms-like tyrosine kinase 3 (*FLT3*) gene are the second most common in AML (~30% of patients) and the most important prognostic factor so far.[128] The most usual polymorphic form is internal tandem duplication (ITD) in the juxtamembrane domain of FLT3, but to a lesser extent point mutations of the tyrosine kinase are also observed.[129] Published data have indicated that FLT3 inhibitors suppress growth of leukemia cell lines and primary AML cells, a fact that is dependent upon expression of mutant FLT3 for growth.[130] Interestingly, one of the most important findings is the existence of a FLT3 mutant form in the LSC population.[131] So far, many compounds have been investigated in preclinical studies and eight of them are now registered for clinical trials.

Tyrphostins (AG1295, AG1296), originally developed as PDGFR and c-KIT inhibitors, were the first inhibitors reported to directly inhibit FLT3 autophosphorylation[132] but never reached clinical trials because of their lack of specificity.[129]

Two indolocarbazoles, lestaurtinib (**6a**, CEP-701, KT-5555, SPM-924, Cephalon)[133] and midostaurin (**6b**, PKC412, Novartis)[134,135] (Figure 6), both staurosporine analogues, were initially identified as tropomyosin receptor kinase A (TrkA) and PKC inhibitors, respectively, but were later recognized as potent

Table 6. Second Generation Bcr-Abl Inhibitors in the Market and in Clinical Development[a]

compd	molecular targets other than Bcr-Abl	T315I inhibition	status
nilotinib (**5b**) (AMN-107, Tasigna), Novartis	-	none	marketed for imatinib resistant CML
dasatinib (**5c**) (BMS-354825, Sprycel), Bristol-Myers Squibb	Kit, PDGFR, ephrin A receptor kinase, and Tec kinase Btk	none	marketed for imatinib resistant CML
bafetinib (**5d**) (NS-187, INNO-406, CNS-9), Nippon Shinyaku	Lyn	none	phase I
bosutinib (**5e**) (SKI-606), Wyeth	Src	none	phase III
XL228, Exelixis	Aurora A, IGF1, Src	positive	phase I
AT9283 (**5h**), Astex Therapeutics	Aurora A and B, JAK-2, JAK-3	positive	phase II
KW2449 (**5g**), Kyowa Hakko Kirin Pharma Inc.	FLT3, FGFR, Aurora A	positive	phase II
PHA-739358 (**5f**), Nerviano Medical Sciences	Aurora	positive	phase II
DCC-2036 (**5j**), Deciphera Pharmaceuticals LLC		positive	phase I/II
homoarringtonine (**5i**), Chemgenex Pharmaceuticals	unknown mechanism of action	positive	phase II
tozasertib (**5k**) (MK-0457, VX-680, VE-465), Merck	Aurora kinase, FLT3, JAK-2	positive	phase II (terminated)

[a] Source: http://www.clinicaltrials.gov/. Abbreviations: CML, chronic myeloid leukemia; FLT3, fms-like tyrosine kinase 3; IGF1, insulin like growth factor 1; PDGFR, platelet derived growth factor receptor.

FLT3 inhibitors. Both these compounds appear to have mediocre effects as a monotherapy, but complete remissions were reached (80−100% of patients) when combined with induction chemotherapy.[128]

Indolinones have been extensively investigated as FLT3 inhibitors, although this activity arose from selectivity studies and the compounds were initially developed as vascular endothelial growth factor receptor (VEGFR) inhibitors. The most studied compounds are SU5416[136] (**6c**) and **6d** (Figure 6), the last being therapeutically used in renal cell carcinoma and imatinib resistant GIST. Both these compounds have inhibitory activity on c-KIT, PDGFR, and VEGFR. **6d** (SU11248, SUTENT, Sugen) was developed on the basis of previous SAR that verified that this molecule exhibited the most optimal overall profile in terms of potency for inhibiting receptor tyrosine kinase targets, solubility, protein binding, in vivo pharmacokinetic properties, and antitumor efficacy.[137] On the contrary, however, the development of **6c** was terminated because of the short half-life of this compound in vivo, a result that gave poor pharmacokinetic properties to the molecule and limited clinical usefulness.[138]

6d as a monotherapy resulted in partial responses of short duration,[139] an effect also observed with **6f** (Figure 6). **6f** (BAY 43-9006, NEXAVAR, Bayer) is marketed for renal cell carcinoma, but the finding that it effectively inhibits FLT3-WT (wild type) and FLT3-ITD (mutated)[140] opened clinical trials for AML patients. Inefficient as a single agent and with activity only for FLT3-ITD AML,[141] it was also studied in phase II trials with induction chemotherapy, and the last registered clinical trial is a combination of G-CSF and Plerixafor plus **6f** (programmed to start on March 2010[14]).

Two more agents were registered for clinical trials but they were discontinued. A phase I clinical trial with dovitinib[142] (**6g**, CHIR-258, TKI258, Novartis, Figure 6) on AML patients was terminated because of time-dependent drug accumulation in the body.[14] The piperazinylquinazoline tandutinib (**6e**) (MLN518, CT53518, Millennium, Figure 6) studied in a phase II trial has shown reduction of peripheral AML blast cells in 30% of patients examined in FLT3-ITD AML but with limited efficacy on patients with FLT3 point mutations.[143] The latter caused discontinuation of the phase II trial.

The compounds that were tested until now were "accidentally" found as FLT3 inhibitors and what was lacking from the area of trials was a selective inhibitor, rationally designed for FLT3 inhibition. AC220 (**6i**) (developed by Ambit Biosciences), a bis-arylurea derivative, is the most recent clinical candidate in the category of FLT3 inhibitors. Discovered from lead optimization of AB-530[144] (**6h**), **6i** (Figure 6) is lacking the carboxamide group and has the addition of a highly water-soluble morpholine in order to exhibit an improved water solubility and pharmacokinetics.[145] When compared to the other FLT3 inhibitors that entered clinical trials, **6i** was the most potent cellular FLT3-ITD inhibitor tested and also had the most enhanced inhibitory effect on cell proliferation along with highly attained selectivity.[146] Apart from FLT3, kinases with binding affinities within 10-fold K_d (1.6 nM) were c-KIT, PDGFRα, PDGFRβ, RET, and CSF1R and within 100-fold K_d were FLT1, FLT4, DDR1, and VEGFR2.[146] When tested in a phase I clinical trial in AML patients, the toxicology profile was acceptable, rendering this compound tolerable and along with the excellent pharmacokinetic profile exhibited in humans and its beneficial clinical effects. The progression of **6i** to phase II was justified.[147,148]

Regarding the structural requirements of SMIs for binding to FLT3 kinase, the limited information is attributed to unsuccessful attempts to cocrystallize a FLT3−inhibitor complex. All the inhibitors discovered so far are ATP competitive based on biochemical assays, indicating the ability of these molecules to bind in the ATP pocket of FLT3.[149] Thus, general observations on structure are a purine/pyrimidine moiety mimicking the adenine structure in ATP and an aromatic domain that binds in the back pocket of the ATP site.[150]

In summary, the AML patients who harbor FLT3 mutations have the greater possibility to benefit from FLT3 inhibitors therapy. Data obtained thus far have shown that FLT3 inhibitors exhibit optimal therapeutic potential when combined with conventional chemotherapy. The sequence of coadministered agents seems important, so that factor should be taken under consideration in clinical trials.[151] Also, focus should be given on pharmacokinetic profile as

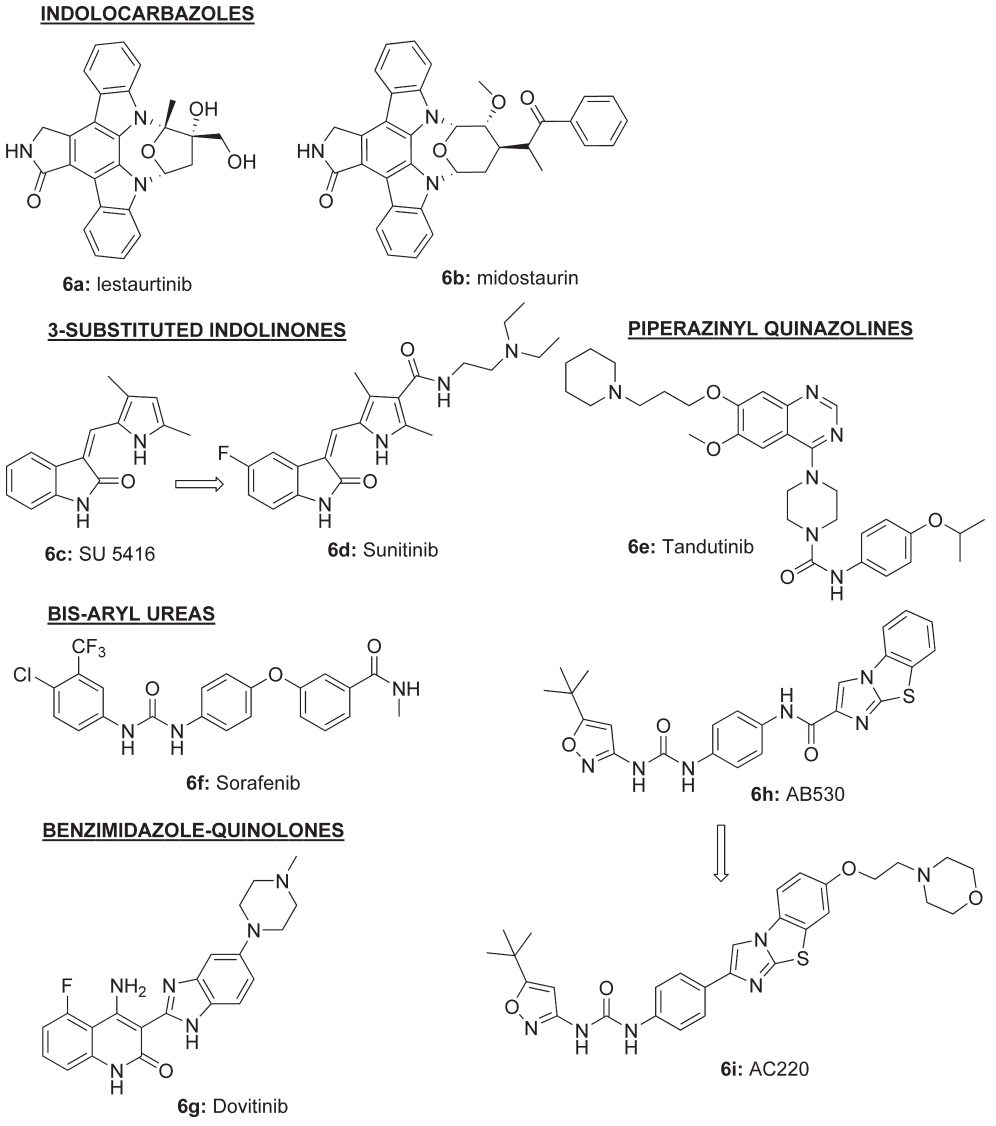

Figure 6. FLT3 inhibitors.

well as toxicity of future inhibitors in order to attain stable therapeutic concentration levels in the plasma over long periods of time, a factor of crucial importance in clinical responses.

6.2.3. VEGFR Inhibitors. In acute leukemia, angiogenesis was identified as another potential therapeutic target.[152] In the most studied leukemia being AML, VEGF and other proangiogenic proteins are involved in its pathophysiology, since VEGF-promoted angiogenesis in bone marrow was positively correlated to AML progression.[153] Compounds that have been examined in clinical trials for leukemias are **6c**, **6d**, vatalanib,[154] pazopanib,[155] and **6f**. Since the main field of investigation for these compounds is solid tumor biology and therapy, only phase I trials have taken place for leukemia with insufficient results.[153] However, combination therapy trials of these compounds with other anticancer agents either of novel mechanism or of conventional chemotherapeutics are still in great demand in oncology.

6.3. mTOR Inhibitors. Rapamycin (**7a**, sirolimus, Figure 7) and its analogues (also called "rapalogues") are the most studied of this field. Initially isolated from *Streptomyces hygroscopicus* in the mid-1970s in a sample collected from the Easter Island Rapa Nui, **7a** was first characterized as a potent antifungal and immunosuppressive agent. Antitumor activity was detected on a NCI screening program taking place in the 1980s, although its mechanism of action was unknown until the mid-1990s when the mammalian target protein of rapamycin (mTOR) was identified. mTOR exists in two forms, mTOR1 and mTOR2.[156] Extensive clinical trials on **7a** delivery for a variety of pathological conditions resulted in its approval by the FDA as an immunosuppressor in kidney transplantation in the late 1990s.[157,158]

Structurally, **7a** is a macrocyclic lactone, including two regions that bind to mTOR and the FK506-binding protein (FKBP12). To be biologically active, **7a** forms a complex with FKBP12, which in turn binds to FKBP-rapamycin-binding domain (FRB) domain adjacent to the catalytic domain of mTOR, thus inhibiting the signaling cascade.[159] The "rapalogues" mostly examined all bear these two regions unmodified with derivatization affecting only the substituent on C-42 as has previously been revealed. Interestingly enough, these compounds interact only with mTOR complex 1 (mTORC1), since the FRB domain in mTORC2 seems to be inaccessible to them.[160] This effect has been blamed for the modest activity of rapalogues in the clinic, since there is feedback activation of PI3K/AKT signaling.[161]

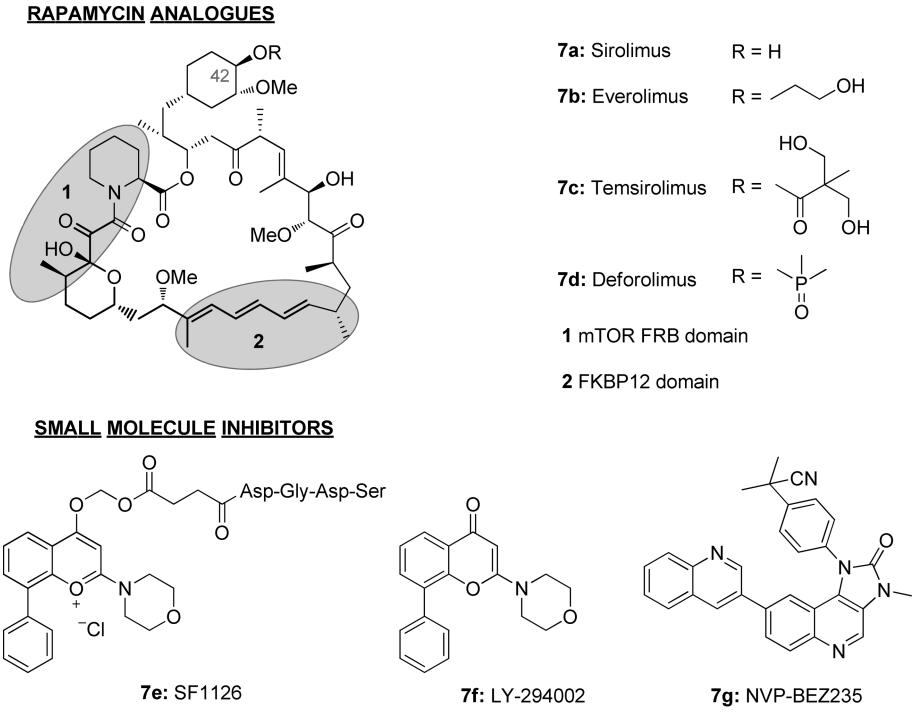

Figure 7. mTOR inhibitors.

The most studied analogues of **7a** are everolimus (**7b**), temsirolimus (**7c**), and deforolimus (**7d**) (Figure 7). All these compounds share great similarity to the parent compound being modified only to the C-42 substituent. **7c**, the 42-[2,2-bis-(hydroxymethyl)]propionic ester of **7a**, has improved water solubility in comparison to the parent compound, and it is the first analogue to be developed. After extensive clinical testing in a number of malignancies, **7c** acquired FDA approval in 2007 for the treatment of advanced renal cell carcinoma. In hematological malignancies it is currently being studied in CLL, AML, CML either alone or in combination with other anticancer agents (Table 7), but the most promising results came from a clinical trial on mantle cell lymphoma with a response rate of 38%.[162] In mantle cell lymphoma cells, mTOR signaling was shown to be activated and contributing to tumor cell survival and progression. On the other hand, mutations on phosphatase and tensin homologue (*PTEN*), which acts as a tumor suppressor gene, were found in most cancers and thought to be responsible for rendering the cells resistant to apoptosis. The same cell population, though, has proved to be significantly more sensitive to sirolimus (**7a**), a fact of clinical importance that needs further investigation in order to explore mTOR and PTEN functions in mutated forms of malignancy.[163,164]

7b, 42-*O*-(2-hydroxyethyl)rapamycin, is an analogue developed for per os administration. It is currently approved as an immunosuppressive agent by FDA for kidney and heart transplantation (CERTICAN), as well as for renal cell carcinoma resistant to treatment with **6d** or **6f** (AFINITOR). **7b** is also undergoing clinical trials for AML and ALL as monotherapy, as well as combination therapy with classical chemotherapeutic agents or epigenetic drugs for AML, MDS, CLL, myeloma, and lymphoma (Table 7). It is of great importance that in AML cells, **7c** and **7b** suppress assembly of mTORC2, inhibiting AKT signaling in vitro and in vivo.[165] As deregulation of the PI3K/AKT pathway is a common feature of hematological malignancies, inhibition of mTOR may provide a useful novel therapeutic strategy.

7d is a dimethylphosphinate rapamycin analogue that retains high affinity to FKBP12 and is also stable in organic solvents, water (regardless of the pH), and plasma. Administration is possible via the intravenous route or per os.[159] Most clinical trials on **7d** have focused on sarcoma and endometrial cancer, but as far as leukemia is concerned, a phase II study in patients with refractory hematologic malignancies has been conducted, showing partial benefit for patients.[166]

There is a plethora of other rapalogues synthesized with precursor-directed biosynthesis, genetic manipulation, and directed mutasynthesis.[167−170] However, since these analogues never reached the clinic, we will not focus on them and examine instead some SMIs of mTOR.

Selective SMIs of mTOR have been synthesized but are still at preclinical levels of evaluation.[171] Thus far, attention has been drawn to mixed mTOR/PI3K inhibitors such as SF1126[172] (**7e**), NVP-BEZ235[173] (**7g**) (Figure 7), and XL-765[174] (**7h**, structure undisclosed) that are currently in phase I clinical trials. **7e** is a prodrug of the morpholinochromenone LY294002 (**7f**, Figure 7) with improved solubility and bioavailability that exhibits pan-PI3K inhibition and is now undergoing phase I evaluation for advanced or metastatic solid tumors.[172] **7g** is an imidazo[4,5-*c*]quinoline that inhibits PI3K/mTOR by binding to their ATP cleft with low nanomolar affinity.[173] Finally, **7h** is also a nanomolar inhibitor of PI3K and mTOR currently in three phase I trials on solid tumors and gliomas.

6.4. Proteasome Inhibitors. The potential role of proteasome in leukemia has been extensively reviewed.[175−177] Following commercialization of bortezomib[178] (**8a**) for the effective treatment of multiple myeloma and cell lymphoma, a number of proteasome inhibitors have been synthesized and, apart from **8a**, four of them [MLN9708[179] (**8b**), salinosporamide A[180] (**8i**), carfilzomib[181] (**8e**), and CEP-18770[179] (**8c**, Figure 8)] are being evaluated in clinical trials on hematological malignancies (mostly in multiple myeloma and lymphoma).

Table 7. Rapalogues (mTOR Inhibitors) in Currently Ongoing Clinical Trials for Leukemia[a]

compd	combination trials	leukemia	phase (NCT ID)[b]
sirolimus (**7a**) (rapamycin, Rapamune), Wyeth	monotherapy	acute leukemia, NHL	phase I (NCT00068302)
	+mitoxantrone +etoposide +cytarabine	AML, CML	phase I (NCT00780104)
	+PEG-asparaginase	ALL	phase I (NCT00957320)
	+corticosteroids	ALL	phase I (NCT00874562)
	+etoposide +cytarabine	ALL, CML	phase I/II (NCT00776373)
	+combination chemotherapy	AML	phase II (NCT00634244)
temsirolimus (**7c**) (CCI-779, Torisel), Wyeth	monotherapy	CLL	phase II (NCT00290472)
		NHL, CLL	phase II (NCT00084474)
	+clofarabine	AML	phase II (NCT00775593)
	+imatinib	CML	phase I (NCT00101088)
	+IMC-A12	advanced or metastatic cancer	phase I (NCT00678223)
everolimus (**7b**) (RAD001, RAD001C, Afinitor, Certican), Novartis Pharma	monotherapy	AML	phase I (NCT00636922)
		ALL	phase I/II (NCT00968253)
	+PKC412	AML, MDS	phase I (NCT00819546)
	+panobinostat	lymphoma, MM	phase I (NCT00962507)
	+bortezomib	mantle cell lymphoma, NHL	phase I (NCT00671112)
	+alemtuzumab	CLL	phase I/II (NCT00935792)
	+nilotinib	AML	phase I/II (NCT00762632)
	+cytarabine +daunorubicin	AML	phase I (NCT00544999)

[a] Source: http://www.clinicaltrials.gov/. Abbreviations: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma. [b] Represents the National Clinical Trials Identifier (NCT ID).

Depending on the structure, the proteasome inhibitors thus far studied can be divided into five classes: peptide aldehydes, peptide boronates, peptide vinyl sulfones, peptide epoxyketones, and β-lactones.[182] Apart from β-lactones, all the other classes of inhibitors are peptides harboring an electrophilic group at one end that can form covalent bonds with the catalytic threonine residue of the proteasome subunits.[183] β-Lactones form adducts. Peptide aldehydes and vinyl sulfones share metabolic instability, lack selectivity,[177] and are not potent candidates for further development and clinical application.

8a (MLN341, LDP-341, PS-341, MG-341, VELCADE, developed by Millenium Pharmaceuticals) is an *N*-acyl-pseudodipeptidylboronic acid and was developed in an effort to replace the aldehyde moiety of proteasome inhibitors. Peptidyl aldehydes are also potent inhibitors of thiol proteases and calpains and display instability due to the acidity of the α-proton. Replacement of the aldehyde with the boronic acid moiety was favorable in terms of potency as well as selectivity, since **8a** displayed IC_{50} = 0.6 nM and a 200 000-fold selectivity for proteasome over cathepsins.[178] Preclinical studies,[184] followed by extensive clinical trials resulted in commercialization and the continuation of clinical trials for a variety of indications. Currently **8a** is included in 453 clinical trials, 41 of which refer to leukemia treatment.[14] A large number of trials that promote combination therapy with **8a** are also under investigation, and some of the most promising for leukemia include combinations with tipifarnib[185] (**9a**), **2a**/**2b**, **1b**, and romidepsin and classic chemotherapy.

8b is a novel proteasome inhibitor also developed by Millenium Pharmaceuticals. It is an orally bioavailable inhibitor that in plasma rapidly hydrolyzes to its metabolite MLN2238, capable of binding reversibly to the chymotrypsine-like subunit of the 20S proteasome.[186] It first entered clinical trials on March 2009 in a phase I trial for advanced nonhematological malignancies. Following that trial, two more phase I trials for lymphoma and multiple myeloma emerged, as well as a phase I/II trial on patients with relapsed or refractory multiple myeloma.[14]

Another boronic acid in clinical development is **8c**. Bearing the pyridylcarboxamide moiety, this dipeptylboronic acid was developed as an oral proteasome inhibitor. It displays selectivity for the trypsin over chymotrypsin activity (more than 1000-fold), and in a panel of 42 protease assays only modest inhibition of cathepsin G and chymase was observed. Cellular permeability is very high (EC_{50} = 13.5 nM in the MOLT-4 cellular assay and IC_{50} = 13.7 nM in the enzyme assay), and oral bioavailability in rats and mice was 54% and 39%, respectively.[179] **8c** is currently in a phase II clinical trial on relapsed multiple myeloma refractory to recent therapy.

Upon delivery of **8a** at recommended doses, limiting toxicity arose that was related to peripheral neuropathy and thrombocytopenia, in part due to the inhibition of serine proteases (although much less in potency, there is parallel inhibition of them). In this context, compounds of alternative chemical structure were sought and a very promising candidate proved to be carfilzomib (PR-171, **8e**). **8e**, a structural analogue of the natural product epoxomicin (**8d**), belongs to the epoxyketone class of proteasome inhibitors. **8e** exhibits potency equal to that of **8a** but greater selectivity for the chymotrypsin-like activity of the protasome. Furthermore, in preclinical evaluation, **8e** exhibited promising tolerability and efficacy that resulted in clinical evaluation trials, up to phase II, as a treatment for hematological malignancies.[181]

As an epoxide, **8e**, is very reactive and its stability was too problematic to allow oral administration. On the basis of such data, accepted oral bioavailability values were warranted, and afterward through a series of structure optimization, the novel inhibitor PR-047 (**8f**) was developed (Figure 8). Though an epoxide, the methoxymethylene chains improved the molecule's solubility and shortening of the peptide part improved bioavailability.[187] **8f** is still in

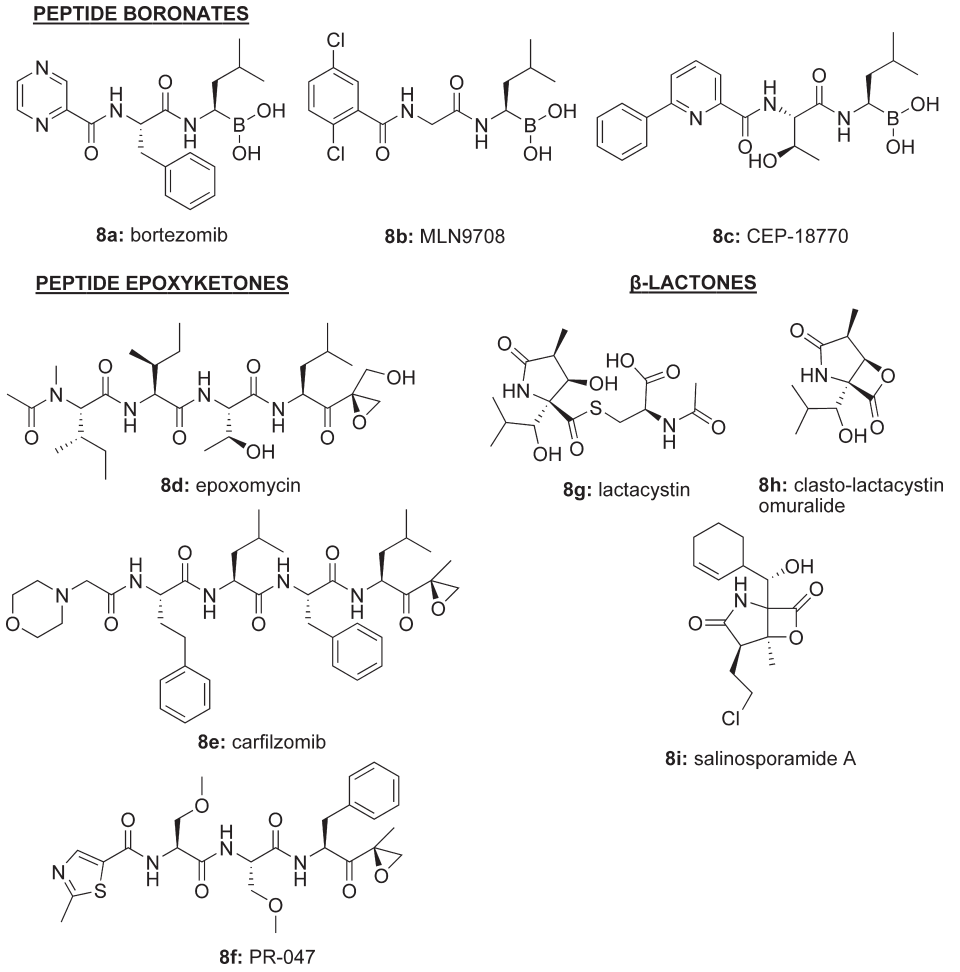

Figure 8. Proteasome inhibitors.

preclinical evaluation, but with IC_{50} = 82 nM and oral bioavailability of 17% (mouse), 21% (rat), and 39% (dog), it has been recommended for clinical development.[188]

From the field of natural products, lactacystin (**8g**) and **8i** are two proteasome inhibitors of the β-lactone class. **8g** is a thioester prodrug, precursor to clasto-lactacystin (or omuralide, **8h**, Figure 8) which is the active β-lactone-γ-lactam.[189] This bicyclic ring system is also found in **8i** but differently substituted and is considered to be the active pharmacophore of this group of compounds after evaluation of hydrolyzed derivatives. **8i** is a product of the marine actinomycete *Salinospora tropica* that acts as an irreversibly bound inhibitor of the proteasome. Moreover, apart from the chymotrypsin-like activity (2.6 nM) it also inhibits trypsin-like activity (21 nM) and to a lesser degree caspase-like activity (430 nM).[180] **8i** is currently investigated in phase I clinical trials for advanced malignancies, relapsed/refractory multiple myeloma, solid tumors, and lymphoma. Also, preclinical studies have shown potential use in CLL,[190] and combination therapy with HDACIs is also conducted in leukemia cells[191] and in a phase I clinical trial (NCT00667082).

The binding kinetics of proteasome inhibitors seems to be of utmost importance in their pharmacological profile. In particular, irreversible inhibitors might be trapped in proteasomes in readily available sights upon their first entrance in the body (e.g., RBCs, liver), a fact that renders lower concentration inhibitors to exhibit therapeutic efficacy in malignant sites ("sink effect").[192] Such disadvantages are considered to be diminished in the cases of **8a** and **8b** as well as **8c** which are slowly and rapidly reversible inhibitors, respectively. However, most studies are needed to verify the pharmacological importance of such an approach.

6.5. Farnesyl Transferase Inhibitors (FTIs). The Ras family of genes encodes proteins implicated in key cellular processes such as proliferation, differentiation, and survival. Ras activation is dependent on isoprenylation (farnesylation catalyzed by FTase or geranylation catalyzed by GGTase) signaling its transport from the cytoplasm to the membrane. *Ras* is frequently deregulated in human cancer. Focusing on hematological malignancies, *Ras* mutations have been observed in 10−65% of cases studied. More specifically, *Ras* mutations have been encountered in 5−15% of patients in ALL, 10−40% in MDS, 15% in AML, and up to 65% in CMML.[193] Moreover, *Ras* activation can occur through other mechanisms such as through the Bcr-Abl chimaeric kinase in CML.[194]

FTIs were first synthesized in an effort to prevent activation of RAS by blocking its post-translational modifications.[130] These molecules prevent the transfer of a farnesyl moiety to a cystein residue (belonging to the CAAX motif; C stands for cysteine, A for aliphatic amino acid, and X for any amino acid) of numerous substrate proteins, RAS included.[195] So far, FTIs are categorized in four classes of inhibitors based on the approach being able to block FTase.[193]

6.5.1. Farnesyl Pyrophosphate (FPP) Analogues. Examples of these agents, such as α-hydroxyfarnesylphosphonic acid, were among the first FTIs to demonstrate inhibition in

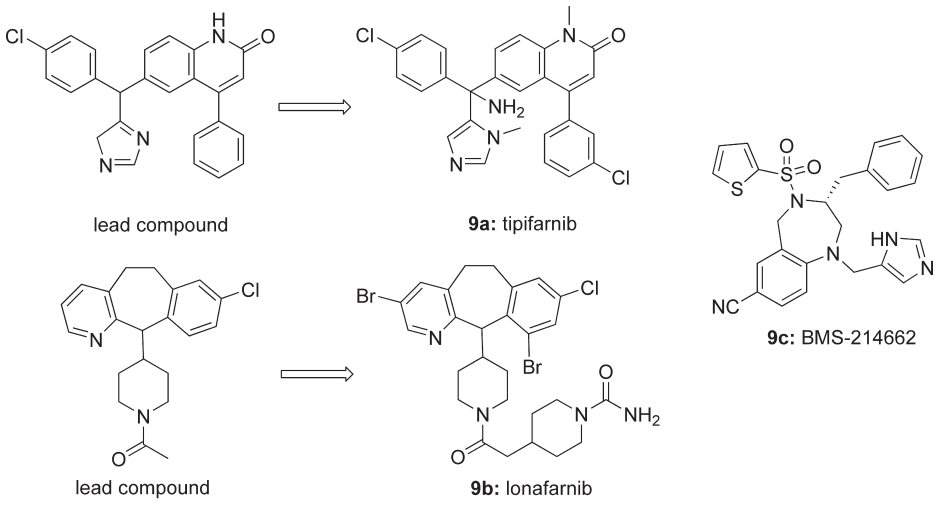

Figure 9. Farnesyl transferase inhibitors (FTIs).

cell culture.[193] However, the design and synthesis of these analogues were swiftly terminated, since FPP is used in multiple normal cellular processes and these analogues could result in excessive toxicity.

6.5.2. CAAX Peptidomimetics. The most promising agent, L-788123, was tested in a phase I clinical trial for advanced solid tumors but was discontinued because of severe toxicity (grade IV thrombocytopenia, fatigue, and cardiac conduction abnormalities).[196] The agents that comprise this category were all extensively ionized at normal pH values and exhibited limited membrane permeability resulting in reduced in vivo activity.

6.5.3. Bisubstrate Analogues. These agents were developed in an effort to simultaneously target both FPP and CAAX. Problematic pharmacokinetic profile, however, was once again displayed, since these agents had poor intracellular infusion due to their large size.

6.5.4. Nonpeptidomimetic Inhibitors. This category includes heterogeneous small molecules belonging to a variety of chemical structures. These non-peptide mimetics share the common feature of competition with the protein−CAAX motif. Since the previous categories of FTIs had unfavorable physicochemical properties resulting in poor pharmacokinetics, the development of such inhibitors was focused on small-molecule non-peptide mimetic agents. The most promising agents evaluated thus far [the orally bioavailable **9a**, lonafarnib[130] (**9b**), and the iv administered BMS-214662[197] (**9c**) (Figure 9)] were further processed in clinical trials and are still under evaluation as a monotherapy or in combination with other anticancer drugs for the treatment of leukemia and other malignancies (Table 8).

9a was the first FTI to enter the clinic in 1997. It is the most extensively studied FTI, counting over 70 clinical trials on various malignancies. In hematologic malignancies it has reached phase III trials either alone or in combination with other anticancer agents. **9a** emerged from structure optimization after an initial computer-assisted screening procedure on antifungal libraries of Janssen. The imidazole ring in its structure seems of utmost importance, being also a common feature with **9c**, targeting the Zn^{2+} ion of FTase.

Having a stereocenter, the (*R*)-enantiomer of **9a** proved to be about 50-fold more potent than the (*S*)-enantiomer, reaching IC_{50} and EC_{50} values of 0.6 and 1.8 nM, respectively.[198] Its good oral bioavailability and linear pharmacokinetic profile contributed to the numerous clinical evaluations conducted thus far. From the very beginning, it was apparent that its use is more beneficial in hematologic malignancies in comparison to solid tumors.[199] Moreover, **9a**'s concentration in bone marrow of leukemic patients appears to be 3- to 4-fold higher than serum levels, making myeloid malignancies the most potent to clinical response.[200] So far, **9a** has been clinically evaluated mostly in AML but also in MDS and CML.[185] The most recent combination clinical trials including **9a** with conventional chemotherapy are in phase III evaluation, and other trials with etoposide and **8a** are in phase II. Combination of **8a** with **9a** has been also advantageous, since it overcomes resistance in multiple myeloma and AML cells in a preclinical survey.[201]

The benzocycloheptapyridyl pharmacophore emerged from studies based on the screening of a library of chemical compounds initially developed as potential antihistaminics by Schering-Plough. Derivatization of the lead compound SCH-37370 resulted in the discovery of **9b**, a compound that, except for its optimal potency ($IC_{50} = 1.9$ nM and COS $IC_{50} = 10$ nM), also had an advantageous pharmacokinetic profile and could be administered orally (76% oral bioavailability).[202] Crystallography studies on the complex of FTase with **9b** inspired the synthesis of indolocycloheptapyridyl compounds[203] that were further optimized to produce a second generation of inhibitors bearing the known moiety of the imidazole. The most potent inhibitor (SCH-226374) exhibited FTase IC_{50} in the subnanomolar range and acceptable pharmacokinetic behavior but did not proceed to clinical testing.[204]

The clinical evaluation of **9b** is centered on progeria (HutChinson-Gilford Syndrome) and solid tumors. Although there are some clinical trials for leukemia therapy, especially in CML, additional studies are required for final conclusions on **9b**'s potency in leukemia to be reached, since the results so far were not extremely promising. To this end, combination trials should also be conducted, since FTIs seem to synergize with other anticancer agents.

9c, the less studied FTI, was derived from SAR studies on a series of compounds bearing the imidazolylmethyltetrahydrobenzodiazepine scaffold. This agent demonstrated an IC_{50} of 1.35 nM, an EC_{50} of 0.025 μM in cells (NIH3T3 SAG assay), and oral bioavailability of up to 56%.[197] However, because of gastrointestinal and liver toxicity observed after its oral administration, most recent studies have used the intravenous route.[195] In preclinical studies, **9c** is

Table 8. Farnesyl Transferase Inhibitors in Clinical Trials for Leukemia[a]

compd	combination trials	leukemia	phase (NCT ID)[b]
tipifarnib (**9a**) (R115777, Zarnestra), Janssen Pharmacutica	monotherapy	leukemia	phase I (NCT00022451)
		advanced hematologic cancer	phase I (NCT00005967)
		AML	phase III (NCT00093990, NCT00093470)
			phase II (NCT00354146, NCT00093418, NCT00048503, NCT00045396, NCT00027872)
			phase I (NCT00101296)
		MDS	phase I/II (NCT00005846)
			phase I (NCT00005845)
		AML, MDS	phase II (NCT00045396)
		AML, CML	phase I (NCT00004009)
		GLL	phase II (NCT00331591, NCT00360776)
	+cytarabine, daunorubicin	AML	phase I (NCT00101153)
	+idarubicin, cytarabine	AML, MDS	phase I/II (NCT00096122)
	+chemotherapy	AML, MDS	phase I (NCT00124644)
			phase II/III (NCT00454480)
	+etoposide	AML	phase I (NCT00112853)
			phase II (NCT00602771)
	+bortezomib	AML, CML	phase I (NCT00383474)
		AML	phase II (NCT00510939)
	+imatinib	CML	phase I (NCT00040105)
lonafarnib (**9b**) (SCH 66336, Sarasar), Schering-Plough	monotherapy	leukemia	phase I/II (NCT00034684)
		CML	phase II (NCT00038597)
		MDS, CMML	phase III (NCT00109538)
	+imatinib	CML	phase I (NCT00047502)
BMS-214662 (**9c**), Bristol-Myers Squibb	monotherapy	acute leukemia, MDS, CML	phase I (NCT00006213)

[a] Source: http://www.clinicaltrials.gov/. Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; GLL, large granular lymphocyte leukemia; MDS, myelodysplastic syndrome. [b] Represents the National Clinical Trials Identifier (NCT ID).

cytotoxic against several cell lines,[205] but in the phase I clinical trial conducted on AML and MDS patients the responses were characterized as "transient" and of "minimal significance".[206] Even though clinical investigations for **9c** ceased, the more recent observations for potential apoptosis induction in myeloma,[207] B-CLL,[208] and CML cells[209] may draw the attention back to the continuation of its development. The latter is of great importance, since selective induction of apoptosis in leukemia cells as well as in LSCs has emerged as a fruitful approach in treating leukemia.[210]

Although designed to block Ras signaling, the finding that **9a** induced apoptosis in myeloma cells in a Ras-independent manner[211] raised skepticism on the mechanism behind the use of FTIs. So far, the molecular targets identified that seem to be implicated in the mode of action of FTIs include farnesylated proteins such as Rho proteins, the PTP-CAAX phosphatase, lamins A and B, and CENPs (further information can be found in Raponi et al.[212]). Also, signaling pathways like the PI3K-AKT and MAPK/ERK, Fas, NF-κB, and VEGF have been correlated to clinical response to FTIs.[213] In conclusion, the results obtained from the clinical trials with FTIs look promising, although the exact underlying mechanism of FTI activity remains uncertain because of the multiple signaling pathways that are dependent on farnesylation. Another field that needs to be further explored is the upcoming resistance to the current developed FTIs,[214] in order to direct the drug design of future inhibitors and define markers to identify patients most likely to respond to this treatment.

6.6. CDK Inhibitors. Targeting of proteins that regulate the cell cycle progression has drawn the attention of pharmaceutical companies in the 1990s. Cyclin-dependent kinases (CDKs) are key cell cycle regulating proteins, and their function is causally related to cell proliferation potential. Very quickly they became a promising target and research on inhibitors began, initially on pan-CDK inhibitors and gradually on more selective ones. Deregulation of CDK functionality is very common (expressed as CDK hyperactivation or endogenous CDK inhibitors inactivation),[215] and CDK inhibitors are being evaluated in a number of trials for their clinical efficacy. Since the literature on CDK inhibitors is vast, only those that have undergone clinical evaluation are mentioned (Table 9).

A number of flavonoids have been found to be effective CDK inhibitors. The more extensively studied are flavopiridol[216] (**10a**) and P276-00[217] (**10c**, Figure 10). **10a** is a semisynthetic compound derived from the natural compound rohitukine (*Dysoxylum binectariferum*) and the first CDK inhibitor to enter clinical trials. Of the many clinical trials that have taken place, the most promising results come from recent studies on CLL[216,218−220] and AML.[221] The scientific interest coming from the CLL studies is the fact that effectiveness was correlated and linked to certain cytogenetic abnormalities [del(17p13) and del(11q22)] related to disease pathophysiology. Indeed, the clinical response was much greater among the patients exhibiting cytogenetic abnormalities, with a percentage of success reaching 40% and 70%, respectively, thus leading regulatory authorities to grant an orphan drug status for the treatment of CLL in 2007.[222] On the other hand, **10a** was combined with Ara-C and mitoxantrone as a therapeutic alternative in AML, where the response rate reached up to 75% and the survival rates for patients significantly increased.

Table 9. CDK Inhibitors in Current Clinical Trials on Leukemia[a]

compd	targeted CDK molecule	conditions	phase (NCT ID)[b]
AG-024322 (**10h**), Pfizer	CDK1, CDK2, and CDK4	advanced cancer	phase I (NCT00147485)
flavopiridol (**10a**) (HL275, L-868275, HMR-1275, NSC-649890, alvocidib), Sanofi-Aventis	pan-CDK inhibitor	acute leukemia	phase I (NCT00470197)
		refractory lymphoma or MM	phase I/II (NCT00112723)
		acute leukemia or CML	phase I (NCT00278330)
		AML	phase II (NCT00795002, NCT00634244)
		AML, ALL, or CML	phase I (NCT00101231)
		CLL or prolymphotic leukemia	phase II (NCT00464633, NCT00098371)
		CLL or small lymphocytic lymphoma	phase I (NCT00058240, NCT00735930, NCT00377104)
P276-00 (**10c**), Nicholas Piramal	pan-CDK inhibitor	MM	phase I/II (NCT00882063)
		mantle cell lymphoma	phase II (NCT00843050)
PD-0332991 (**10d**), Pfizer	CDK4 and CDK6	advanced cancer	phase I (NCT00141297)
		mantle cell lymphoma	phase I (NCT00420056)
		MM	phase I/II (NCT00555906)
SCH 7727965 (**10e**), Schering-Plough	CDK1, CDK2, CDK5, and CDK9	advanced cancer	phase I (NCT00871663, NCT00871910)
		AML, ALL	phase II (NCT00798213)
		mantle cell lymphoma, CLL	phase II (NCT00871546)
SNS-032 (**10f**) (BMS-387032), Synesis	CDK2, CDK7, and CDK9	B-lymphoid malignancies	phase I (NCT00446342)

[a] Source: http://www.clinicaltrials.gov/. Abbreviations: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; CML, chronic myeloid leukemia; MM, multiple myeloma. [b] Represents the National Clinical Trials Identifier (NCT ID).

10c belongs to a group of synthetic flavones studied mostly on solid tumors but recently registered for a phase I/II trial on multiple myeloma (MM) and a phase II trial on mantle cell lymphoma. **10c** is a more potent CDK inhibitor compared to **10a** (up to 3-fold antiproliferative activity in human cancer cell lines), with nanomolar affinity to CDK1, CDK4, and CDK9 and micromolar affinity to CDK2, CDK6, and CDK7.[217,223]

Another important class of CDK inhibitors is purine-based compounds. Olomoucine was one of the first inhibitors identified and the lead compound for the synthesis of roscovitine.[224] The (*R*)-isomer of roscovitine[225] (seliciclib, CYC202, **10b**, Figure 10) was found to be more potent and entered clinical trials on solid tumors, although with no encouraging results due to its pan-CDK inhibitory activity. The analogues purvalanol A[226] and NU6140[227] are in preclinical development as the more recent pyrazolotriazine bioisoster N-&-N1 (GP0210).[228] Because they are more potent but have no particular selectivity, their potential clinical usage is still under question.

The pyrimidine scaffold also seems to be of critical importance in developing efficient CDK inhibitors. On the basis of former observations on pyrido[2,3-*d*]pyrimidin-7-ones, PD-0332991[229] (**10d**, Figure 10) emerged as a selective CDK4 and CDK6 inhibitor (IC_{50} of 11 and 16 nM, respectively).[230] **10d** is currently studied in phase I and phase II clinical trials either as a monotherapy or in combination with **8a** and dexamethazone. Also of the pyrimidine core and under clinical evaluation is the pyrazolo[1,5-*a*]pyrimidine SCH 727965[231] (**10e**) that has been found to effectively inhibit CDK1, CDK2, CDK5, and CDK9. This CDK inhibitor is currently included in four clinical trials regarding hematological malignancies (monotherapy or combination therapy), but results from these trials have yet to be published in order to evaluate its potential clinical use.

The aminothiazole SNS-032[232] (BMS-387032, **10f**, Figure 10) is an inhibitor of CDK2, CDK7, and CDK9 and is currently in a phase I clinical trial on B-lymphoid malignancies (CLL, mantle cell lymphoma). Additionally, its mechanism of action in CLL is being elucidated as stated by Chen et al.[233] In particular, the poor bioavailability of this compound was attributed to its ability to act as a substrate of P-gp, a fact that allowed scientists to generate new advanced analogues with improved permeability and lower efflux.[234,235]

Identified from fragment-based screening, the pan-CDK inhibitor AT7519[236] (**10g**, Figure 10) is in phase I clinical trials on advanced solid tumors and non-Hodgkin's lymphoma (NHL). Its affinity is higher for CDK2, CDK4, and CDK5 (47, 67, and 18 nM, respectively).[236] In another phase I clinical trial in advanced cancer, the indazole AG-024322[237] (**10h**, Figure 10) was found to be inadequate for differentiating from other pharmaceutic interventions and the trial was terminated.

CDK inhibitors that are currently in clinical trials for cancer therapy, other than leukemia, are the pan-CDK inhibitor of the diaminopyrimidine scaffold R547[238] (**10i**, Ro-4584820) (phase I trials on solid tumors) and the 4,5-dihydro-1*H*-pyrazolo[4,3-*h*]quinazoline PHA-848125[239] (phase II trial on thymic carcinoma) (**10j**, Figure 10). The last is a potent orally bioavailable pan-CDK inhibitor that exhibits slightly higher affinity for CDK2 but is also active against CDK4, CDK5, CDK7, with additional good water solubility.[239] Also under preclinical evaluation are the 3-aminopyrazole PNU-292137[240] (**10k**, Figure 10) and the selective CDK1 inhibitor RO-3306[241] (**10l**, Figure 10).

Since CDK inhibitors were tested in clinical trials for more than a decade and none advanced to the market yet, mostly because of mediocre response rates and significant toxicities, the effectiveness of this therapeutic intervention was questioned. In order to proceed more efficiently, the more thorough elucidation of the functions that each CDK is responsible for within the cell should be verified, whereas the genetic characterization of various cancer cell phenotypes that could be potential targets for specific CDK inhibition should be further exploited.[242] Another aspect

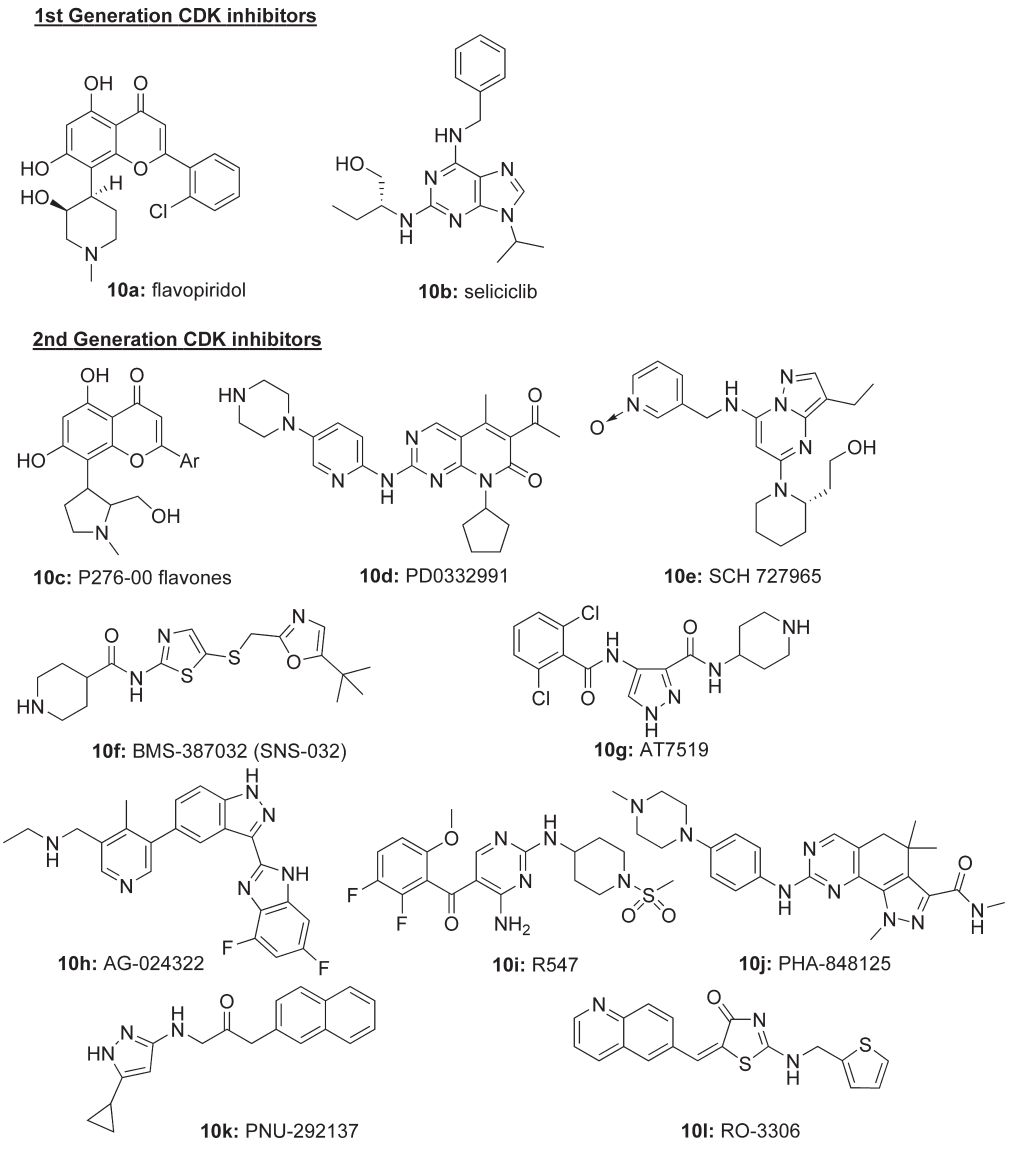

Figure 10. Cycline dependent kinase (CDK) inhibitors.

that also needs attention while evaluating CDK inhibitors for their clinical potential is their pharmacokinetics. This is why sufficient exposure throughout the cell cycle should be guaranteed with a reasonable route of administration because of the existing tumor cell heterogeneity.[243]

6.7. Other Apoptosis Promoting Agents. Aurora kinase inhibitors, as well as heat-shock protein 90 (HSP90) inhibitors, are currently in clinical trials for evaluation on leukemia and other malignancies, as reviewed elsewhere.[244,245] Furthermore, compounds targeting signaling pathways that are under clinical evaluation for leukemia are PF-04449913 (Hedgehog inhibitor)[14] and the γ-secretase inhibitor MK0752 (targeting Notch).[246] Targeting X-linked inhibitor of apoptosis proteins (XIAPs), the small molecule AEG40826 and the antisense oligonucleotide AEG 35156 are currently in clinical trials. The last has advanced to phase II in combination with chemotherapeutics for the treatment of AML.[247] Results seem very encouraging especially in the combination trial, since the complete response rate in patients with AML refractory to single agent chemotherapy reached 91%.[248]

The skepticism on specific enzyme targeting and its efficiency on a heterogeneous disease such as cancer has led researchers to seek alternative routes in fighting the disease. Multitarget kinase inhibitors, combination of agents that act through different signaling pathways or combination of conventional with novel therapeutics, are under investigation; such attempts have already been described above. Apart from multitarget kinase inhibitors, the simultaneous inhibition of different signaling pathways with one molecule is a strategy under consideration, though such an approach still has not been evaluated in the clinic. Such an example comes with CU-201 (developed by Curis), a multitargeted inhibitor of HDAC, Abl, and Src.[249] Another novel proposed strategy is targeting of signaling networks instead of specific enzymes. The molecular chaperone HSP90 is such a nodal protein implicated in multiple signaling networks. In addition, its participation in mitochondria homeostasis permitted Kang and Altieri to target HSP90 in that cellular compartment[250] as a means to increase selectivity. In particular, combinatorial chemistry proved to be a useful tool and their compounds (gamitrinibs whose chemical structures consist of a mitochondria targeting part, a linker, and an HSP90 inhibitor) have proven to be successful in xenografted human tumor cell lines in mice. However, this approach is

still in its infancy, so more studies are needed to further evaluate the therapeutic potential.

7. Future Directions for Antileukemic Research

7.1. Therapeutic Strategies To Specifically Target LSCs. The discovery of potential molecular targets as well as the development of more effective and less cytotoxic anticancer drugs still remains a highly challenging endeavor almost 35 years after the "war on cancer" was declared.[251] To overcome the main obstacles hampering anticancer drug discovery, it is crucial to elucidate and thoroughly understand the molecular complexity and cellular heterogeneity seen in tumor cell initiation and progression.[12] Experimental evidence accumulated since 1997 supports the cancer stem cell hypothesis, meaning that a specific cell population within the tumor has the capacity for self-renewal as well as the proliferative potential to maintain and expand the population of malignant cells.[252−254] Cancer stem cells remain resistant and invulnerable to standard chemotherapy because of their quiescent state, while they express typical markers of stem cells. It is still not clear if cancer stem cells originate from genetically or epigenetically deregulated normal stem cells or by dedifferentiation of somatic tumor cells to the stemlike counterparts. Data published by Blair et al.[3] and by Bonnet and Dick[4] showed that a subpopulation of purified leukemic cells ($Thy1^-$, $CD34^+$, $CD38^-$) injected into NOD/SCID mice caused leukemia histologically similar to that of the donor. If leukemias can be considered as a HSC disorder or a reacquisition of HSC characteristics, it can then be expected that many signaling pathways classically associated with leukemia initiation and progression also regulate normal HSC development.[253]

Normal hematopoiesis requires complex and highly orchestrated interactions between the bone marrow microenvironment (or niche) and HSCs, a process that may be deregulated upon leukemia in a way to cause perturbation of self-renewal of HSCs and support of self-renewal of LSCs.[255,256] Therefore, the development of innovative leukemia therapeutics must be based on the molecular pathways and targets regulating survival and self-renewal of both HSCs and LSCs. These include HSC niche components, signaling pathways (SCF/c-kit-R, EPO-R-JAK2/STAT, Wnt, Notch, HOX), inducer−receptor interactions, superfine chromatin-structure modifications, fused transcription factors, microRNAs, and signaling of apoptosis, as presented elsewhere.[1] Moreover, recent data indicate the importance of PTEN tumor suppressor and the phosphatidylinositol 3-OH-kinase (PI3K) pathway in LSCs formation.[257] Interestingly enough, it has been shown that *Pten*-gene loss in mouse HSCs through the activation of β-catenin pathway and via multiple genetic and molecular alterations contributes to LSC transformation and expansion.[258] These data were further strengthened by observations showing that β-catenin is essential for survival and self-renewal of LSCs insensitive to **5a** treatment in mice with Bcr-Abl-induced CML, thus providing a new strategy for targeting these cells.[248] Recent observations also revealed the proliferative effects of the cytokine interferon-α (IFNα) on LSCs and the importance of eliminating resistance to chemotherapy LSCs in leukemia patients. In particular, it has been indicated that although dormant LSCs are resistant to antiproliferative agent 5-fluorouracil (5-FU), LSCs pretreated with IFNα, and thus exit cell dormancy and induced to proliferate, are efficiently eliminated by 5-FU exposure in vivo.[259] The latter support the notion of the potential therapeutic application of IFNs in shifting quiescent LSCs into the cell cycling state to increase their sensitivity to cytotoxic agents.[260]

The identification and purification of LSCs along with the establishment of their characteristics are expected to provide future powerful diagnostic, prognostic, and therapeutic tools in the clinical oncology of leukemias. Moving forward, the identification of molecular mechanisms underlying the behavior of HSCs and LSCs will permit better understanding of how leukemia is initiated and allow more efficient treatment options by innovative bifunctional and multitargeted medicines, impinging on crucial molecular networks and targets that regulate execution decisions of LSCs to self-renew, proliferate, differentiate, and/or undergo apoptosis. To this end, by using genomic approaches to identify potential key regulators of HSC quiescence and leukemogenesis, researchers were able to identify a subset of genes that may contribute to leukemic development from LSCs.[261] Alternatively, detailed understanding of the interactions occurring between normal hematopoietic and leukemic cells within the bone marrow microenvironment can uncover unknown mechanisms that reflect the emergence of drug resistance and heterogeneity seen in leukemic cells.[1,10,262]

7.2. Pharmacogenomics and the Development of New Antileukemia Drugs. Identification of novel drug-exploitable molecular targets allowed the development of new molecularly designed anticancer medicines. However, the emergence of rapidly acquired drug resistance to limit clinical efficacy of recently developed chemotherapeutics along with tumor genetic variability and microenvironmental factors suggested that pharmacogenomics-related drug response variations may also contribute to the clinical outcome of cancer patients.[7,103] Furthermore, the concept of genetically based antileukemia drug development is further strengthened by the fact that individual patient genetic makeup also affects the clinical outcome and alters the strategy of drug delivery toward the so-called personalized cancer medicine. Therefore, it becomes obvious that the study and exploitation of interindividual genetic variations are crucial to understand why some antineoplastic agents are safe and effective in some patients but not in others. This means that the assessment of germ line polymorphisms and host pharmacogenomics of specific disease-related as well drug pharmacodynamics- and pharmacokinetics-related genes is now considered as an important approach in developing new antileukemia molecularly targeted medicines and in assessing their efficacy and safety in clinical practice.[7,263]

Clinical translation of drug-related genetic information has allowed the analysis of genetic variations of drug-metabolizing enzymes, transporters, and receptors on a routine basis as well as the correlation of such data with altered drug-related responses.[263−265] Technological achievements in DNA microarray methodologies allowed the recording of gene expression profile data in clinical samples that predict a patient's clinical outcome to a given or more than one therapeutic agent.[266] Indeed, leukemia was one of the first diseases in which such an approach was applied in order to analyze the effects of **3a** on genes and pathways involved in the so-called differentiation therapy of APL.[267] Another example of pharmacogenomics in leukemia is one of thiopurine *S*-methyltransferase (TPMT), an enzyme involved in the deactivation of 6-mercaptopurine (6-MP) and thioguanine (TG) used for the treatment of childhood ALL. Interindividual genetic variations of the *TPMT* gene were the first to prove the clinical value of individualizing drug dosage to achieve a better clinical outcome (a specific personalized

medicine approach).[265,268] Numerous pharmacogenomic studies have indicated that low TPMT enzyme activity is well correlated with the observed hematopoietic toxicity of thiopurine drugs in clinical practice. Three variant alleles of the *TPMT* gene (*TPMT*2*, *TPMT*3A*, *TPMT*3C*) were found to account for more than 95% of the inherited variability in TPMT enzyme activity. This finding has permitted genetic identification of patients at high risk of toxicity after thiopurine therapy. Indeed, clinical evidence now indicates that some TPMT-deficient patients that are homozygous in one TPMT gene variant require a reduction of more than 90% of the conventional dose of thiopurine drugs in order to respond efficiently without toxicity in this therapy.[269]

Another example related to pharmacogenomics of leukemia therapeutics refers to methotrexate (MTX) delivery in childhood ALL treatment protocols. As has been shown, the uptake of MTX within ALL cells is primarily mediated by the transporter SLC19A1, whereas its cellular efflux is mediated by the ATP-binding cassette transporters (ABC transporters) like ABCC2.[270] Such processes contribute toward the achievement of therapeutic intracellular MTX concentrations being able to inhibit its target enzyme dihydrofolate reductase (DHFR) and thus to exert its antileukemia effect. Specific mutations in either the *ABCC2* transporter gene or folate-dependent enzymes 5,10-methylenetetrahydrofolate reductase (MTHFR) and thymidylate synthase (TYMS) have been shown to influence MTX treatment response in childhood ALL.[269] Recently, the synergistic effect of specific TPMT and MTHFR genotypes on the observed 6-MP toxicity in patients with childhood ALL has also been clinically evaluated.[271]

Although the prospects for basic research in pharmacogenomics and the generation of considerable amounts of data look very promising, their incorporation in new drug development and clinical practice is quite challenging.[263] Furthermore, by assessment of the actions of drugs at the molecular level, it has been clearly shown that medicines exert their effects via specific "molecular networks" involving several genes and proteins.[2,27,272] The genetic background involved in the response of an organism to delivered drugs is complex and is also determined by the interaction of genes with drugs. This interplay between genes and drugs also implies that their interaction can modulate the pharmacotherapy outcome through either genetic variation or drug-regulated gene expression that consequently leads to phenotypic variation, e.g., differential pharmacological response. The complexity underlying the gene expression profile in crucial cellular decisions related to leukemogenesis is recently outlined by shedding light on the transcriptional network that controls growth arrest and differentiation in human myeloid leukemia cells.[273] Also, concerning the clinical use of epigenetic drugs in MDS to modulate gene expression and affect cell growth, differentiation, and apoptosis, such complexity emerged at the molecular level, since it has been recently shown that DNA methylation inhibitors azacitidine, **2b**, and **2c** exert differential effects on the cancer gene expression profile in AML cells.[274] Interestingly enough, the methylation inhibitors neplanocin A and 3-deazaneplanocin A (DZNep) block the in vitro differentiation program of murine erythroleukemia (MEL) cells by activating silent DNA regions,[275] whereas DZNep reactivates developmentally regulated genes by inhibiting histone methylation.[276] In addition, it is now well-known that gene expression can also be affected by environmental factors, like epigenetic phenomena including DNA methylation, histone acetylation, and RNA interference (RNAi) mechanisms that represent crucial mechanisms for establishing specific gene expression patterns in cellular physiology.[5,39,277] The latter might also contribute into interindividual variability seen in drug response, since both environmental and genomic factors may finally modulate drug efficacy and cause toxicity in the body. As a matter of fact, the design of pharmacogenomics studies to analyze the molecular mechanisms underlying drug response variability must be taken into consideration upon attempting to develop innovative anticancer drugs.

8. Concluding Remarks

Experimental evidence accumulated over the past several years has indicated that human leukemias represent disorders of self-renewal, growth, differentiation, and/or apoptosis of HSCs or their early multipotent progenitors. The discovery of LSCs as LICs indicates that human leukemias may arise from such immortalized cells that share specific properties with normal HSCs. It has been said that LSCs are quiescent and thus less vulnerable to conventional antiproliferative agents. LSCs represent quite heterogeneous cell populations that exhibit different degrees of sensitivity to leukemia therapeutics. Therefore, other innovative multifunctional and multilevel target antileukemia agents are desperately needed to eradicate LSCs and their transformed progeny.

The fact that leukemia cells regardless of their genetic abnormalities and the acquired multidrug resistance (MDR) are able to regulate their renewal capacity, proliferation, differentiation, and apoptosis at different levels (signaling, growth and proliferation response stimuli, cell cycle-dependent kinases, proteasome activation, etc.) suggests that LSCs (or LICs) cannot be affected by treatment with conventional cytotoxic agents but by targeted therapies on specific sites (e.g., an enzyme) acting at different levels. Multilevel targeting can be done either by using classes of agents that complement each other under a combination chemotherapy approach or by developing bi- or multifunctional agents promoting or blocking more than one vital process of leukemic development. Agents able to block self-renewal, halt cell growth, promote differentiation, and provoke apoptosis can be useful for effective leukemia therapy.

This paper reviews the basic principles of leukemia cell differentiation and apoptosis with emphasis on differentiation inducers, hybrid/polar compounds, HDACIs, DNMT inhibitors, retinoids and retinoid mimetics, Bcl-2 inhibitors, regular and chimeric tyrosine kinase inhibitors, FLT3 kinase inhibitors, VEGFR inhibitors, mTOR inhibitors, proteasome inhibitors, FTIs, CDK inhibitors, and classes of agents developed and under clinical evaluation. The potential role of pharmacogenomics in the development of these new antileukemia agents is comprehensively discussed in a way to achieve improved clinical outcomes through the application of personalized medicine concepts in cancer therapy.

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Biographies

Ioannis S. Vizirianakis is currently an Associate Professor in Molecular Pharmacology and Pharmacogenomics at Aristotle University of Thessaloniki, Greece. After receiving his degree in Pharmacy (1981) from Aristotle University of Thessaloniki, he joined the department as Research Assistant (1985−1990), obtained a Ph.D. (1991) in Biochemical Pharmacology under Prof. Tsiftsoglou, and finally was assigned as Lecturer (1993)

and then Assistant Professor (2003) in Pharmacology. He received a research grant award (1998) from Cancer Coordinating Committee upon his sabbatical at the Tumor Biology Laboratory, University of California, San Francisco. His long-term research interests focus on "differentiation therapy of cancer" and include the development of innovative chemical inducers of differentiation of leukemia cells through the understanding of crucial molecular mechanisms that govern leukemia cell initiation, unrestricted growth as well as blockade of differentiation, and apoptosis.

Maria Chatzopoulou received her degree in Pharmaceutical Sciences in 2006 and her M.Sc. in Pharmaceutical Chemistry in 2008 from Aristotle University of Thessaloniki, Greece. Currently she is a Ph.D. candidate in the Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Aristotle University of Thessaloniki, with a scholarship from the Greek State Scholarship Foundation and under the guidance of Prof. V. J. Demopoulos. Her research activities involve drug design and organic synthesis of pharmaceutical compounds. She has published papers in peer reviewed journals and attended several conferences presenting her results.

Ioannis D. Bonovolias received his degree in Pharmaceutical Sciences (B.Pharm.) from Aristotle University of Thessaloniki, Greece, in 2005. Then he carried out graduate studies (2005−2007) in Pharmacology and Therapeutics at the same department and received his M.Sc. degree in 2007 on the mechanisms of drug resistance to antileukemia drugs. He is currently a Ph.D. candidate and under the supervision of Prof. A. S. Tsiftsoglou since 2003. He has published papers in peer reviewed journals and attended several international conferences presenting his results.

Ioannis Nicolaou received his degree in Pharmaceutical Sciences from Aristotle University of Thessaloniki, Greece, in 1995. He then started his graduate studies at the Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Aristotle University of Thessaloniki under the guidance of Dr. V.J. Demopoulos, leading to a doctorate degree in 2000. He continued his postdoctorate studies from 2000 to 2004 at Aristotle University of Thessaloniki under a variety of grants. Currently, he is a Lecturer at the Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Aristotle University of Thessaloniki, from September 2005. His research activities involve drug design and synthesis of compounds that combine aldose reductase enzyme inhibitory activity and ability to prevent the nonenzymatic irreversible modification of proteins from monosacharides. Recently, the design and synthesis of derivatives with antileukemic activity entered his field of interest.

Vassilis J. Demopoulos received his degree in Pharmacy from the University of Athens, Greece (1977). He then joined the School of Pharmacy, University of Iowa, as Research Assistant and obtained his Ph.D. in Pharmaceutical Chemistry and Natural Products under Prof. J. G. Cannon (1982). He joined the Department of Chemistry, University of Newfoundland, Canada, as Research Associate (October 1981 to August 1982). In 1985, he returned to Greece, joining the Department of Pharmacy, Aristotle University of Thessaloniki, Greece, as Lecturer (1985−1990), Assistant Professor (1990−2003), Associate Professor (2003−2009), and Professor (2009−present). He is a member of The European Federation for Pharmaceutical Sciences, the Division of Medicinal Chemistry of the American Chemical Society, the Panhellenic Association of Pharmacists, and the Hellenic Society of Medicinal Chemistry (executive committee). His research activities involve drug design and synthesis of bioactive compounds.

Asterios S. Tsiftsoglou received his degree from the Department of Pharmacy of Aristotle University of Thessaloniki (1972), Greece, and earned his M.Sci., M.Phil. (1978) and completed his Ph.D. (1979) at Yale University, Department of Pharmacology, under Prof. A. C. Sartorelli. He did postdoctoral research at Massachusetts Institute of Technology (CCR) (Cambridge, MA) (1978−1981) and then served as Assistant Professor at the Harvard Medical School (Beth Israel Hospital, Hematology-Oncology) (Boston, MA) (up to 1986). He returned to Aristotle University of Thessaloniki where he teaches Molecular Pharmacology and Biotechnology. He is Professor of Pharmacology and has been Chairman several times. His major research is on leukemia cell differentiation. He is member of several scientific societies, reviewer in peer-reviewed journals, and core member of committees (CPWP, BWP, CAT) in EMEA. He has published close to 100 original papers and chapters in peer reviewed journals and books.

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