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Differentiation of cancer stem cells using synthetic small molecules: toward new therapeutic strategies against therapy resistance

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Abstract

Despite the existing arsenal of anti-cancer drugs, 10 million people die each year worldwide due to cancers, highlighting the need to discover new therapies based on innovative modes of action against these pathologies. Current chemotherapies are based on the use of cytotoxic agents, targeted drugs, monoclonal antibodies or immunotherapies that are able to reduce or stop the proliferation of cancer cells. However, tumor eradication is often hampered by the presence of resistant cells called cancer stem-like cells or cancer stem cells (CSCs). Several strategies have been proposed to specifically target CSCs such as the use of CSC-specific antibodies, small molecules able to target CSCs signaling pathways or drugs able to induce CSCs differentiation rendering them sensitive to classical chemotherapy. These latter compounds are the focus of the present review that aims at reporting recent advances in anticancer differentiation strategy. This therapeutic approach was shown to be particularly promising for eradication of tumors in which CSCs are the main reason for therapeutic failure. This general view of the chemistry and mechanism of action of compounds inducing the differentiation of CSCs could be particularly useful for a broad range of researchers working in the field of anticancer therapies since the combination of compounds that induce differentiation with classical chemotherapy could represent a successful approach for future therapeutic applications.

1. Introduction

In recent years, the World Health Organization (WHO) highlighted that cancer is responsible for the death of almost 10 million people worldwide each year.^[1] This suggests that even if an arsenal of anticancer drugs exists, its efficacy requires further improvement. In order to develop new effective therapeutic agents, a better understanding of the biology and hallmarks of cancer became fundamental.^[2]

Cancer cells are the foundation of the disease since they express oncogenic and tumor suppressor mutations thus defining cancer as a genetic disease and are able to initiate tumors and induce tumor progression.^[3] The hallmarks of cancers include various items such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. In addition, while cancers have been long considered as homogeneous tissues, recent evidences demonstrated the presence of intratumor heterogeneity and in particular of subclasses of neoplastic cells called cancer-initiating cells, cancer stem-like cells or cancer stem cells (CSCs).^[4] This added a further level of complexity in the understanding of tumor biology. CSCs were first identified twenty years ago in acute myeloid leukemia.^[5-6] A small subset of cancer cells was capable of driving tumor progression and promoting tumor growth in a mouse model resulting in the development of the CSCs model.^[7-8] CSCs have been functionally defined thanks to their ability to efficiently induce the formation of new tumors and based on the expression of specific markers.^[9] The exact origins of CSCs still need to be completely elucidated and probably vary from one tumor type to another. Indeed, normal tissue stem cells could be the cells of origin giving rise to CSCs upon oncogenic transformation, but it is also possible that progenitor cells, i.e. partially differentiated cells, could undergo oncogenic transformation assuming a stem-like character.^[10] Also, the concept of CSCs is closely related to epithelial-mesenchymal transition (EMT) that enable cancer cells to disseminate from primary tumors to other tissues.^[11] EMT also confers to cancer cells the self-renewal capability that is essential for their clonal expansion at sites of dissemination.^[12] It is important to note that, analogously to what happens during EMT, the plasticity present within tumors induce the bidirectional interconversion between CSCs and non-CSCs, resulting in dynamic variation in the relative abundance of CSCs.^[13] This phenotypic plasticity is an important feature of CSCs that may enable the formation of functionally distinct subpopulations within a tumor thus supporting overall tumor growth in various ways. Altogether these parameters highlight the great complexity of tumor biology that needs to take into account also the contribution of the tumor microenvironment (TME).

The presence of CSCs in several types of cancer is believed to be the main cause of failure of chemotherapy and radiotherapy.^[14] Despite the difficulties to precisely define CSCs because of their intrinsic plasticity, CSCs generally share the following features: (i) unlimited self-renewal capacity since they are able to produce progeny cells that are identical to the parental cells and that are able to

maintain tumors; (ii) differentiation potential since they can produce different lineages of differentiated tumor cells; (iii) high tumorigenicity since a small number of CSCs cultured *in vitro* is able to form colonies and few CSCs are required to form tumors *in vivo* upon injection; property that is not shared by non-CSC tumor cells; and (iv) general resistance to chemotherapy and radiotherapy. The main factors for the development of therapy resistance are as follows. First, most CSCs are in a resting or dormant state and are not undergoing cell division. Second, CSCs mostly express ATP-binding cassette family membrane transporters, which are responsible for the transport and efflux of metabolites, drugs, toxic substances, endogenous lipids, peptides, nucleotides and sterols thus rendering these cells resistant to many chemotherapeutic drugs. Finally, the abnormal expression of signaling pathway components and the diversification of CSCs microenvironment are also related to drug resistance.^[15]

Thanks to a better understanding of human tumor biology, various treatments have been developed to supplement surgery and radiotherapy. In the past decades, anticancer treatments have considerably progressed not only with new cytotoxic agents, but also with the design of targeted drugs, monoclonal antibodies or immunotherapies. All these anti-cancer therapies showed successful results but failed in some cases to cure entirely the tumor thus giving rise to drug resistance and recurrences. In this context, the design of novel therapeutic approaches that could lead to effective inhibition and suppression of tumor growth and relapse is needed. Since CSCs are one of the reasons of therapeutic failure, a number of studies have suggested that the targeting of CSCs could overcome resistance.^[16-18]

Various strategies have been developed to tackle resistance phenomena with therapies that kill CSCs, inhibit specifically the maintenance of the stem-cell state or induce differentiation of cancer stem cells. Two main classes of therapeutic tools have been used to this aim: (i) antibodies to target cell surface and transmembrane proteins specific of CSCs or signaling pathways involved in CSCs development and maintenance, (ii) small-molecule drugs targeting specific signaling cascades, inhibiting efflux pumps often responsible for CSCs resistance or inducing the differentiation of CSCs thus rendering them sensitive to chemotherapy.^[19] While the use of antibodies has shown very promising results and some of them are currently in clinical trials, approaches based on the use of small molecules able to target specific features of CSCs and induce the elimination of these aggressive and tumorigenic cells have led to major therapeutic progresses with drugs currently approved or in clinical trials.^[18] The use of small molecules to take advantage of CSCs inherent plasticity and induce the differentiation of CSCs could render these cells less tumorigenic and, more importantly, sensitive to chemotherapy. This differentiation strategy is particularly promising for the treatment of cancers that cannot be treated with classical therapies and because they are expected to bear a larger therapeutic index than classical cytotoxic approaches.

Various reviews recently described the general strategies developed so far to specifically target CSCs.^[19-22] In this review, after a short summary of the previously reported validated approaches to target CSCs already described in these previous reviews, we will focus on the description of small

molecules that have been demonstrated to induce the differentiation of CSCs together with their mechanism of action. This subject has not been reviewed so far and we intend here to illustrate the strategies developed to identify such compounds, the diversity of their applications and the potential of the differentiation approach that holds the promise for future efficient chemotherapies.

2. Specific targeting of CSCs

CSCs bear not only major genetic and epigenetic differences compared to differentiated cancer cells but also marked phenotypic variations such as specific cell surface markers and signaling pathways that can be targeted to affect CSCs proliferation.^[18] Various cell surface and transmembrane proteins are overexpressed by CSCs while absent in other cancer cells and normal tissues. These are the main tools that enabled the isolation of human CSCs from heterogeneous tumors by fluorescence-activated cell sorting or magnetic bead isolation and they can be used for the identification of such cells.^[23] To make a non-exhaustive list, CD44, CD47, CD123 (CD standing for Cluster of Differentiation), ALDH (Aldehyde dehydrogenase), EpCAM (Epithelial Cell Adhesion Molecule), TGF β (Transforming Growth Factor beta) or IGF (Insulin-like Growth Factor) receptors are among the most known and studied markers. However, there are a number of other markers that have been identified and such cell surface markers are not universal, even within the same tumor type, thus rendering their identification and targeting complicated.^[24]

Also, many signaling pathways exist in CSCs and their activation promotes the evolution and regulates the maintenance and survival of CSCs. Their involvement in cancer development varies with the type of cancer but three signaling pathways remain the most investigated due to their role in self-renewal, proliferation and differentiation mechanisms of CSC: Canonical Wnt, Notch and Hedgehog (Hh) pathways. Wnt pathway is involved in cell proliferation, plays a role in normal embryonic stem cell development and is also linked to carcinogenesis in adult tissues.^[25] Notch is involved in embryogenesis, in the development of several organs during fetal development as well as angiogenesis and bears critical roles in adult stem cell maintenance.^[26] Hedgehog (Hh) is a crucial signaling pathway in embryonic stem cells and is involved in a large number of cellular and molecular mechanisms including cancer development.^[27] In most cases, inappropriate activation of these pathways stimulates proliferation, inhibits differentiation and prevents apoptosis. Blocking these pathways may thus provide new avenues for cancer therapeutic strategies based on CSCs targeting.^[28]

Beside cell surface markers and signaling pathways, niches are specific regions of the tumor microenvironment (TME) where the CSCs reside and where the TME exerts its maximum influence. TME supports the equilibrium of cancer stem cells behavior regarding initiation, growth, maintenance, self-renewal, differentiation, metastasis and therapeutic resistance.^[29] TME has three major features: (i) chronic inflammation and secretion of inflammatory cytokines, (ii) hypoxia and (iii) perivascular niches that regulate the capacity of proliferation and differentiation. Blocking or influencing these

features holds the potential for multi-CSC targeting purpose since tumours of different origin share common niche elements.^[30]

Finally, it has been demonstrated that CSCs bear an upregulated iron metabolism since they extract iron from the microenvironment more effectively than other tumor cells and preferentially require transferrin receptor and ferritin, two core iron regulators, to propagate and form tumors *in vivo*. Iron is a unique, primordial metal fundamental for earliest life forms, on which CSCs depend and this dependence can also represent a specific target for CSCs.^[31]

As mentioned in the introduction, two main therapeutic tools have been applied to the targeting of these specific CSCs features reaching, in some cases, clinical trials: antibodies and small molecules. Monoclonal antibodies directed against cell surface markers were shown to exhibit significant anti-CSCs activity.^[32] As a representative example, Catumaxomab is currently in clinical trials as anti-EpCAM antibody against non-small cell lung cancer.^[33] Antibodies have also been developed against CD44 or CD123 and some of them are currently in phase II clinical trials for different types of cancers.^[34-35] Furthermore, antibodies have been used to target signaling pathways. Demcizumab was developed against Notch pathway and Fresolimumab against TGF β and both are in phase I/II clinical trials.^[36-37] It is now clear that antibodies have great potential for the specific targeting of CSCs but still present some limitations for their therapeutic application, such as bad pharmacology as well as toxicity and side effects. For these reasons, other approaches based on the use of small druggable compounds have been developed in the field of CSCs targeting.

Interestingly, various small molecules have been identified for their ability to target specifically CSCs acting on cell surface markers or signaling pathways as previously described for antibodies, but also as inhibitors of drug efflux pumps, tumor microenvironment (TME) and CSCs niches or apoptotic pathways (*Figure 1*).^[17,38-39] Some of these drugs are currently in clinical trials for their ability to affect efficiently different types of cancer *via* these mechanisms of action. For example, two small-molecule inhibitors of Hh pathway were approved by FDA: Vismodegib, first approved in 2012 and developed by Genentech, and Sonidegib, approved in 2015 and developed by Novartis, are used to treat basal cell carcinoma.^[40-41] Both compounds target the Smoothed homolog receptor (SMO) for Hh inhibition and are under clinical trials for the treatment of other solid tumors such as NCT02111187 for prostate cancer, NCT02027376 for breast cancer and NCT02195973 for recurrent ovarian cancer. CXCR1/2 inhibitors such as Reparixin or CXCR4 inhibitors such as Plerixafor, both in phase II clinical trials, have shown efficacy in combination to chemotherapeutic agents when employed to target the interactions between CSCs and the microenvironment.^[42-43]

As mentioned above, one of the main mechanisms of drug resistance of CSCs is related to the overexpression and high activity of ATP-binding cassette (ABC) transporters that represents a promising target to sensitize CSCs to anti-cancer therapies.^[44] From a historical point of view the most widely studied ABC transporter is P-glycoprotein (P-gp), mainly for its involvement in drug resistance that widely occurs in bacteria, viruses and cancer. The feasibility of ABC transporters targeting in

CSCs has been demonstrated with salinomycin, a polyether antibiotic isolated from *Streptomyces albus* that has been shown to act either as a specific inhibitor of CSCs and as a potent inhibitor of P-gp by the induction of a conformational change of the protein.^[45-46] It is important to note that salinomycin bears other mechanisms of action against CSCs such as iron homeostasis upon sequestration of lysosomal iron that is essential for CSCs survival.^[47] Other evidence of the efficacy of inhibiting ABC transporters for the targeting of CSCs also came from metformin, an anti-diabetes II drug.^[48] This compound was shown to reduce the CSCs population upon repression of a microRNA: miR-27b. This latter inhibits ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1) that induced the generation of CSCs *via* upregulation of an ABC transporter. Metformin thus interferes with this cascade and eventually reduces the expression of ABC transporters. Even more importantly, metformin showed also to decrease glucose levels by activating AMP-activated protein kinase (AMPK) in hepatocytes resulting in a reduced activity of acetyl-CoA carboxylase and an induction of fatty acid oxidation.^[49] Recently, it has been demonstrated that metformin accumulates in mitochondria directly reducing copper levels in cancer cells similarly to salinomycin for iron levels.^[50] This led to mitochondrial stress and apoptosis. It has to be noted that mesenchymal cancer cells depend on copper and that metformin inhibits EMT in these cells. Mitochondrial copper thus represents also a promising target in cancer.

The elimination of CSCs based on the re-activation of apoptosis, a mechanism that mediates survival and death in each cell, has also been investigated since this mechanism is impaired during cancer development and in CSCs. Some therapeutic agents could indeed activate apoptosis pathways and lead, in combination with anticancer agents, to CSCs eradication. This is the case of Bortezomid, a dipeptidyl boronic acid compound that reversibly blocks the proteolytic activity of the proteasome and induces glioblastoma stem cells death in a synergistic manner with TRIAL (tumor necrosis factor-related apoptosis-inducing ligand).^[51] Another transcription factor linked to the control of apoptosis, NF- κ B, has also been targeted by therapeutic agents in order to induce cell death.^[52] As an example, Parthenolide was shown to inhibit NF- κ B pathway in breast cancer, preferentially affecting stem cells thus further demonstrating that NF- κ B is crucial to maintain the survival of CSCs.^[53]

This short summary of the main approaches used to target CSCs shows that a large number of researches are devoted to this end and that clinical trials are currently in progress to lead these therapeutic tools to clinical applications. It is worth mentioning that beside antibodies and small molecules other innovative approaches are explored. For example, oncolytic viruses have been studied for the targeting of CSCs since they are not recognized by ABC transporters and they are able to affect some cellular pathways involved in tumor development.^[54] Various oncolytic viruses have been used for cancer therapy including adenovirus, poxviruses, herpes viruses as well as some RNA viruses. In this context, capsid-modified oncolytic viruses that can enter both proliferating and quiescent cells through infection were developed.^[55] Oncolytic viruses act with a variety of mechanisms of action and in general viral replication leads to oncolytic death of the cell, with the release of thousands of virions

that mediate effective intratumoral penetration and dissemination to distant tumors. As an example, adenoviruses are effective in killing breast cancer stem cells *in vitro* and *in vivo* but do not lead to complete tumor eradication.^[56] Also, the use of oligonucleotides to interfere with microRNAs in cancer has also been developed as an approach to tackle the presence of CSCs.^[57] MicroRNAs are small non-coding RNAs responsible for the regulation of gene expression and they are involved in many biological processes such as cell proliferation, differentiation and apoptosis. Levels of microRNAs are different in a CSC or in a non-tumorigenic cell and also differ depending on the type of tumor. It has been shown that microRNAs are involved in CSCs maintenance and that they modify several signaling pathways that could transform stem cells and differentiated cells into cancer stem cells.^[58] Oligonucleotides have thus been used to supply for under-expressed tumor suppressors microRNAs or to directly inhibit the action of oncogenic ones and succeeded in specifically targeting CSCs functions.^[59]

Altogether, these approaches demonstrated that targeting CSCs is a very promising approach toward tumor eradication with a large range of applications. However, specific effects on CSCs are not completely elucidated and combination therapies are necessary to obtain efficient results. Furthermore, a limited number of targets have been explored so far as demonstrated by the limited number of tools that have reached clinical trials. Thus, even if all these different approaches to specifically target CSCs are promising and could lead to the desired therapeutic effects, innovative research is needed to further improve clinical applications. One of the most recent strategies to tackle CSCs tumorigenicity and therapy-resistance is the search for small-molecule compounds that could induce differentiation of CSCs rendering these cells sensitive to the cytotoxic action of known anticancer compounds. Differentiation therapy could induce CSCs to lose their tumorigenic potential and to increase their sensitivity to classical chemotherapy thus leading to tumor eradication when used in combination with classical chemotherapy. The differentiation approach is thus distinct, even if complementary, to the strategies presented above. This original therapeutic approach is still in its infancy but holds the promise for future efficient therapies. Noteworthy, the therapeutic applications of such strategy would be extremely large. The focus of this review is to describe current approaches for the induction of the differentiation of CSCs using small natural or synthetic compounds. In the following sections, we will describe the examples of small molecules bearing differentiation ability on CSCs that have been reported so far with the corresponding mechanisms of action and applications as well as the methodology that led to their discovery. This should be useful for future studies in the field and for developing new medicinal chemistry studies toward innovative anticancer therapies.

3. Induction of CSCs differentiation

Among the different anti-CSC therapeutic strategies developed so far, the differentiation approach aims at the modification of the CSCs population induced by exogenous intervention that would render

these cells non tumorigenic and sensitive to chemotherapies. The main goal of this approach is to act on the plasticity (i.e. the ability of one cell type to convert into another across lineage) by forcing CSCs to differentiate and lose their self-renewal and drug resistance properties.^[60] The possibility to transform CSCs could represent an innovative and efficient therapy and thus raises as a promising way to eradicate various kinds of cancers. The idea of differentiating cancer stem cells has received a lot of support due to favorable results from preclinical and clinical studies in treating acute promyelocytic leukemia by differentiation therapy with retinoic acids (such as all-trans retinoic acid or ATRA, a vitamin-A analog).^[61] One approach to induce the differentiation of CSCs is to affect specific signaling pathways that control cell proliferation and differentiation with small molecules that are routinely used to manipulate these pathways. This kind of compounds can be identified through several approaches, but the most widely employed is the high-throughput screening of chemical libraries such as FDA-approved drugs, inhibitors of signaling pathways or natural products. This has been particularly performed in search for cytotoxic compounds specific for CSCs such as the screening that led to the identification of salinomycin as a CSCs specific inhibitor.^[62] Depending on the type of screening, this approach can be limited by the need for *a priori* knowledge of the molecular pathways involved in biological processes of interest. This is particularly problematic in stem cell biology where biological mechanisms are still poorly understood. Despite this limitation, different studies have shown that differentiation therapies are possible and represent promising approaches for future cancer therapies. In the sections below, we will overview the approaches used to induce CSCs differentiation using small molecules and their outcomes toward the discovery of more specific and efficient compounds with clinical applications.

3.1 All-trans retinoic acid (ATRA)

The all-trans retinoic acid (ATRA), also called Tretinoin (compound **1**, *Figure 2*), is an active metabolite of vitamin A and a member of the retinoid family. Structurally, retinoids possess a β -ionone ring and a polyunsaturated side chain, with either an alcohol, an aldehyde, a carboxylic acid group or an ester group. The side chain is composed of four isoprenoid units, with a series of conjugated double bonds that may exist in trans- or cis- configuration.

Retinoids recognize two distinct families of nuclear receptors: retinoic acid receptors (RARs) and retinoid receptors (RXRs), each class including three isoforms, α , β and γ . Found in human plasma and in other tissues, ATRA regulates a variety of essential biological processes *via* gene-regulatory mechanisms. Indeed, the actions of retinoids are mediated through the nuclear retinoid receptors controlling transcription of different sets of genes of cell differentiation depending on the cell type. Retinoids, including retinoic acid, retinol, retinal and retinyl ester, exert potent effects on cell growth, differentiation and apoptosis and showed significant results in cancer therapy and chemoprevention.^[63] ATRA chemical structure was elucidated in 1931 and 50 years later, in the early 1980s, it was discovered that ATRA was able to induce differentiation of human promyelocytic leukemia cells *in*

vitro.^[64] Thus, concomitantly with the growing interest in the CSCs theory, ATRA was deeply investigated as a CSCs differentiation agent.

First of all, ATRA was clinically approved for the treatment of patients affected by acute promyelocytic leukemia (APL) in 2007. APL is characterized by a t(15;17) chromosomal translocation resulting in a fusion protein of retinoic acid receptor (RAR) and promyelocytic leukemia gene, and all-trans retinoic acid (ATRA) leads to terminal differentiation of the myeloid blasts.^[65] ATRA is thus used in clinic in combination with arsenic trioxide for APL treatment with high therapeutic efficacy.^[66]

ATRA was also evaluated for its effects on other cancers, such as on glioblastoma, an incurable brain cancer, where CSCs play a major role in resistance to chemotherapy and therapeutic failure. It was demonstrated that ATRA is able to induce the differentiation of glioblastoma stem cells (GSCs).^[67] The biological assays performed on GSCs showed that upon treatment with ATRA a pronounced morphological change occurs, GSCs-derived neurospheres quickly attach to the culture flask, branch out and lose their neurosphere-like shape thus suggesting differentiation of these cells. However, complete differentiation was not achieved and recurrence was observed. Investigations about head and neck squamous carcinoma CSCs (HNSC CSCs) showed that ATRA induces differentiation of these cells as suggested by significant reduction of stem cell markers Oct4, Sox2, Nestin and CD44.^[68]

ATRA also contributes to chemosensitization of HNSC CSCs to cisplatin and suppresses proliferation of these CSCs *in vitro* and *in vivo* thanks to the inhibition of the Wnt/ β -catenin pathway that results in the decrease of HNSC CSC stemness. ATRA also induces similar effects on the differentiation of CSCs in hepatocellular carcinoma (HCC).^[69] Stemness markers, such as CD133, CD90, and CD24, were significantly reduced and expression of a cluster of liver-specific genes was increased on ATRA-treated cells supporting the differentiation postulate. Moreover, improvement of chemotherapeutic effect of cisplatin was observed when this cytotoxic compound was combined with ATRA, due to elimination of CSCs *via* ATRA-induced differentiation. Even if the molecular mechanism of action of ATRA on CSCs differentiation has not been completely elucidated, it has been suggested that the combination of ATRA with cisplatin could inhibit anti-apoptotic proteins of the protein kinase B (Akt)/Induced myeloid leukemia cell differentiation protein (Mcl-1) signaling pathway, which was responsible for the strong cytotoxic effect of the combination treatment.^[69] It was also demonstrated that RXR and RAR receptors are overexpressed in colon cancer CSCs (ALDH+ cells) and ATRA treatment induced the expected differentiating effect in these cells.^[70] This demonstrates that the biological activity of ATRA in CSCs could be also mediated by these receptors. Recently, a study on the gastric carcinoma (GC) has described the effect of ATRA treatment on the tumorigenic properties of CSCs from GC cells.^[71] The authors showed that ATRA affects cellular morphology, inhibits cellular proliferation and downregulates the expression of the CSCs markers as well as some stemness genes, while upregulating the expression of gastric epithelial differentiation markers such as Mucin 6, Cytokeratins, Osteopontin and TFF3 (Trefoil factor 3). These results indicate that ATRA targets GC CSCs properties, affects their self-renewal, survival and tumorigenic properties and induces their

differentiation. This biological activity was confirmed *in vivo*. However, a 2-week ATRA treatment was not sufficient to fully eradicate CSCs, as post-treatment relapses were observed in all cases after 14-28 days. To solve this problem, combination of ATRA with other chemotherapeutic agents has been proposed.^[72]

Even if the induction of differentiation of CSCs with ATRA have shown very promising results in various types of cancer, it is clear that complete differentiation and permanent remission is hard to attain. Synthetic analogs of ATRA have thus been designed to improve the differentiation activity, such as, for example, retinoic acid-triazolyl derivatives bearing various alkyl- and aryl substituents (**2a-d**, *Figure 2*) that were studied on neuroblastoma cells.^[73] Treatment of neuroblastoma cells with some of these new compounds increases typical neuronal differentiation markers such as NF-H and NeuN. Thus, these compounds could act as potential leads in inducing neuronal differentiation for future studies. Since most retinoids are highly lipophilic compounds with propensity to accumulate in human tissues leading to toxicity, less lipophilic retinoic acid analogs were explored in order to use them in the clinic. Tazarotene, Am80P, LG100268 or WYC-209 were studied as ATRA analogs for their effects on CSCs showing the ability to inhibit the proliferation of these cells but were deprived of the differentiating activity of ATRA.^[74-76]

The studies about ATRA and its analogs are still in progress and this class of compounds needs to be further studied for the improvement of the efficacy when employed alone or when used in combination with classical chemotherapy. However, these compounds allowed for a better understanding of CSCs differentiation induced by small molecules and represent an important proof of concept for the differentiation approach.

3.2 Calcitriol (Vitamin D3)

Calcitriol, also known as 1,25-dihydroxycholecalciferol or vitamin D3 [1,25(OH)₂D₃] (compound **3**, and proposed mechanism of action based on the interaction with nuclear receptors RAR and RXR.

Figure), is an active metabolite of vitamin D. Discovered in 1971 with the work of De Luca, Kodicek and Norman,^[77-78] the first differentiation-inducing activities of this molecule were reported ten years later.^[79]

Apart from the known activity on calcium homeostasis and bone mineralization, calcitriol showed biological effects on proliferation, apoptosis, differentiation, inflammation, invasion, angiogenesis and metastasis by modulating multiple signaling pathways.^[15,80] The effects of calcitriol are mediated by the nuclear vitamin D receptor (VDR) which heterodimerizes with the retinoid X receptor (RXR) upon calcitriol binding. This VDR-RXR complex binds vitamin D responsive elements (VDRE) and leads to the regulation of gene expression. For example, calcitriol inhibits cell growth and induces apoptosis in *in vitro* cultured ovarian cancer cell lines.^[81-82] Calcitriol treatment can preferentially activate the VDR signaling pathway in CSCs, which further inhibits the Wnt pathway and disrupts CSCs stemness,

leading to a reduction of the CSC population.^[83] Furthermore, calcitriol was shown to reduce the number of CSCs in thyroid cancer inducing a morphological change toward more differentiated cells.^[84] Gemini vitamin D analog BXL0124 (compound **4**, *Figure 3*) induced the repression of CD44 expression *in vivo* and *in vitro*, probably *via* VDR- and p53-dependent mechanisms in breast cancer.^[85] It was thus suggested that BXL0124 could be a useful agent for repressing CD44-expressing cancer stem cells.

Together with retinoic acids, Vitamin D and its analogs represent promising scaffolds for the discovery of compounds inducing CSCs differentiation with a wide range of applications and could be the starting point for the development of new compounds bearing differentiation activity.

3.3 Smac (second mitochondria-derived activator of caspases) mimetics

Inhibitors of apoptosis proteins (IAP) represent a family of antiapoptotic proteins that is overexpressed in cancer cells and that prevents them from entering the apoptosis process.^[86] IAP are considered promising targets for the development of new anticancer compounds. Among the therapeutic strategies that have been designed to target IAP proteins, the most widely used approach is based on mimicking the IAP-binding motif of second mitochondria-derived activator of caspase (Smac), which functions as an endogenous IAP antagonist.^[87] Endogenous Smac homodimerizes through an extensive hydrophobic interface and is bivalent. Thus, potent and selective Smac mimetic compounds have been developed and some of them are already in clinical trials as drugs that broadly target all IAP. These antagonists are monovalent or bivalent peptidomimetics and include for example TL32711 (Tetralogic Pharmaceuticals, compound **5**, *Figure*) and AT-406 (Ascenta Therapeutics, compound **6**, *Figure*). These inhibitors mimic the *N*-terminal part of endogenous Smac protein and can inhibit tumor growth and proliferation suggesting the induction of CSCs differentiation.

TL32711, also called Birinapant, is able to induce apoptosis in various types of cancers.^[88] Furthermore, it was demonstrated that this compound sensitized tumor-initiating cells in high-grade serous ovarian cancer (HGSC) to carboplatin resulting in the elimination of these CSCs and in a significant increase in disease-free survival.^[89] An analogous effect was observed for both TL32711 and AT-406 in colorectal adenocarcinoma and ovarian cancer.^[90-91]

BV6 (compound **7**, *Figure*) was used to elucidate the mechanism of IAP antagonism. After investigation of the role of BV6 in the sensitization of primary cultured glioma cells as well as in glioblastoma-initiating cancer stem cells for γ -irradiation-induced apoptosis through NF- κ B activation,^[92] Fulda's team revealed the non-apoptotic role of BV6 in the regulation of GBM CSCs differentiation.^[93] The authors evaluated the morphological changes of cells lines, the levels of differentiation markers such as GFAP and β -III tubulin protein as well as the downregulation of stem cell markers and the involvement of NF- κ B signaling pathway in this treatment. These experiments led to the confirmation that at non-cytotoxic concentrations, BV6 promotes differentiation of GSCs upon

activation of NF- κ B pathway and reduces the tumorigenicity of GSCs *in vivo* thus significantly increasing survival in mice.

Altogether, these findings show that Smac mimetics also affect the regulation of CSCs differentiation thus opening new perspective in their use as cancer therapeutics.

3.4 Histone deacetylase inhibitors (HDACi)

Histone acetylation and deacetylation tightly control histone functions and belong to a large group of post-translational modifications that are essential in the regulation of gene expression and for the maintenance of cellular homeostasis. Abnormalities in histone deacetylation are recognized as a hallmark of cancer and the expression of histone deacetylases is frequently altered in several malignancies.^[94] Various broad-spectrum HDAC inhibitors (HDACi) have thus been proposed as an alternative strategy to conventional treatments.^[95] Some of these inhibitors were reported to have suppressive activities or differentiation effects on the CSCs population through different mechanisms. They were shown to induce differentiation in several hematological malignancies as well as in endometrial stromal sarcoma cells, liver cancer cells, small-cell lung cancers and breast cancer. In this section, we will focus on three HDACi that can induce CSCs differentiation: Abexinostat, MC1742 and suberoylanilide hydroxamic acid (SAHA).

Abexinostat (compound **8**, *Figure 5*) belongs to the class of hydroxamic acids HDAC inhibitors and was discovered in 2006 with the development of small-molecule inhibitors of HDACs derived from *N*-hydroxy-benzamide.^[96] Indeed, Abexinostat, formerly PCI-24781, bears a benzofuran amide linked to the *N*-hydroxybenzamide together with the hydroxamic acid function essential for HDAC inhibition activity. This molecule is able to inhibit multiple HDAC isoforms and showed good *in vivo* efficacy. Its pharmacokinetic profile suggests adequate drug exposure to allow HDAC inhibition *in vivo* and it is currently in clinical trials. It was reported that Abexinostat reduces CSCs population in breast cancer cell lines (BCLs) through the induction of differentiation.^[97] In this work, a series of BCLs demonstrated different reactions to Abexinostat in different lineages. Sensitivity to Abexinostat was measured by monitoring the long non-coding RNA Xist expression (Xinactive specific transcript). Indeed, low expression of Xist was observed in sensitive cell lines while an overexpression of this RNA was observed in non-sensitive cells. The low-dose sensitive breast cancer cell lines exhibited important morphologic changes upon treatment with Abexinostat since the size of the cells increased concomitantly with a decreased nuclear/cytoplasmic ratio, suggesting that Abexinostat induces CSCs differentiation in this type of breast cancer cells whereas an apoptotic effect was observed on the high-dose sensitive BCLs. The long non-coding RNA Xist (Xinactive specific transcript) can thus be identified as a biomarker that can predict the BCL response to HDACi. MC1742 (compound **9**,

Figure) belongs to the class of uracil-based hydroxyamides (UBHAs) HDAC inhibitors.^[98-100] These compounds bear aryl/arylalkyl groups linked to a uracil moiety through a sulfur atom and a hydrophobic spacer (C2 to C7 carbon atom chains or methylenecinnamyl groups) bearing the hydroxamate function. MC1742 and its analogs have raised as a class I/IIb-selective HDACi, being potent at submicromolar/nanomolar level against class I HDACs (HDAC1–3 and 8) and at nanomolar level against class IIb HDACs (HDAC6 and 10) as shown by *in vitro* assays.^[98] It was also demonstrated that MC1742 was able to induce cell differentiation of MG-63 osteosarcoma CSCs in a dose dependent manner at non-toxic concentrations.^[91] Similarly, SAHA (also called Vorinostat, compound **10**, *Figure 5*) is a hydroxamic acid that showed the ability to induce differentiation of CSCs. As an example, it was demonstrated that SAHA was able to induce autophagy of glioblastoma stem cells but also that it functioned as a potent modulator of differentiation at reduced doses.^[101] Altogether, these results open promising perspectives for the use of HDACi as differentiation therapy targeting the CSCs population of various cancers. However, some issues need to be solved before these effects could be validated. Indeed, the mechanism by which these HDAC inhibitors suppress the CSC population has not been fully elucidated due to the complexity of the antitumor mechanism of HDAC inhibitors. Likely, HDAC inhibitors interfere with one or more CSC pathways through inhibition of one or more HDAC isoforms. But it is probable that these compounds act also directly on these signaling pathways as demonstrated not only for Vorinostat^[101] but also for Valproic acid^[102] and Trichostatin A^[103] (compounds **11** and **12**, respectively, *Figure*). Furthermore, it remains unclear which of the numerous isoforms of HDACs are responsible for the suppressive effect of pan-HDAC inhibitors on CSCs. Based on these results, further preclinical and clinical studies need to be performed to better disclose the mechanisms by which HDACi modulate CSC signaling pathway.

3.5 Isocitrate dehydrogenase (IDH) inhibitors

In eukaryotic metabolism, isocitrate is converted to alpha-ketoglutarate (α -KG) thanks to its oxidative decarboxylation catalyzed by the enzyme isocitrate dehydrogenase (IDH).^[104] The IDH enzymes bear a key role in the regulation of normal cell growth and differentiation since they are involved in the regulation of many α -KG-dependent dioxygenases, as well as in the protection of cells against reactive oxygen species. Somatic mutations in the IDH1 and IDH2 isoforms (mIDH1, mIDH2) have been described in many tumors prompted efforts to develop small molecule inhibitors of the mIDH enzyme as a therapeutic strategy.^[105] mIDH induce reduction of α -KG to produce (R)-2-hydroxyglutarate (2-HG) that exerts oncogenic effects in various ways, such as the competitive inhibition of α -KG-dependent dioxygenases such as DNA and histone demethylases, which modulate transcription of many genes important in cell differentiation.^[106] Inhibitors of mIDH were developed based on the hypothesis that selective targeting of the mIDH enzyme would block 2-HG production, resulting in an appropriate methylation state and the onset of cell differentiation. Among the most efficient inhibitors,

Enasidenib (AG-221, compound **13**, *Figure 6*), a selective inhibitor of mIDH2, ivosidenib (AG-120, compound **14**, *Figure 6*), a selective inhibitor of mIDH1, and AG-881 (compound **15**, *Figure 6*), a brain-penetrant inhibitor of mIDH1 and mIDH2, are all undergoing clinical evaluation with either mIDH advanced hematologic malignancies (NCT01915498, NCT02074839, and NCT02492737) or mIDH advanced solid tumors, including gliomas, cholangiocarcinomas, or chondrosarcomas (NCT02273739, NCT02073994, and NCT02481154). The chemical structures of **13** and **15** are composed of an aryl ring system in the 6-position of the triazine along with two aliphatic amines in the 2- and 4-positions of the scaffold, while compound **14** is a substituted phenyl-glycine derivative. Combinations of this kind of inhibitors with chemotherapeutic agents are also under clinical studies further demonstrating the feasibility of this strategy and the success of the differentiation approach.

3.6 Dihydroorotate Dehydrogenase (DHODH) inhibitors

DHODH is a mitochondrial enzyme that converts the dihydroorotate (DHO) to orotate which is the fourth step during *de novo* pyrimidine synthesis. The relevance of this enzyme in differentiation and cancer cells was discovered after a high-throughput phenotypic screening performed in a cellular model of acute myeloid leukemia (AML) with acquired resistance to the differentiation to identify compounds able to overcome differentiation blockade.^[107] The active compounds, such as MLR390 (compound **16**, *Figure 7*) were indeed inhibitors of DHODH and were able to trigger myeloid differentiation *in vitro* and *in vivo* thus leading to the reduction of leukemic cell burden, the decrease in levels of leukemia-initiating cells and to improve survival.^[107-108] Even if the mechanism through which a reduction in *de novo* pyrimidine biosynthesis modulates myeloid differentiation is not clear, inhibitors of DHODH are already in clinical trials for AML and showed a general effect in a large range of leukemia cell types. One of the DHODH inhibitors currently in clinical trials is BAY2402234 (compound **17**, *Figure 7*) that shows a selective low-nanomolar inhibition of human DHODH enzymatic activity. *In vitro*, it potently inhibits proliferation of AML cells in the sub-nanomolar to low-nanomolar range and it induces differentiation at the same range of concentrations. BAY 2402234 also exhibits strong *in vivo* anti-tumor efficacy in monotherapy in several subcutaneous and disseminated AML xenografts as well as AML patient-derived xenograft (PDX) models.^[109] DHODH inhibitors thus represent extremely promising tools for the differentiation of AML cells further validating the differentiation strategy for clinical application.

3.6 Metformin

Metformin (compound **18**, *Figure 8*) is a *N,N*-dimethyl-biguanide discovered in 1922 and it is the first-line medication for the treatment of type 2 diabetes. Well-tolerated by the human body, it is on the World Health Organization's List of Essential Medicines and during recent years it also received

attention as a potential anticancer agent. This started in 2005 when Evan et al. reported a correlation between metformin treatment for diabetes and a reduced incidence of tumor formation in various cancer types.^[110] Since then, numerous studies have reported the anti-tumor and anti-CSCs effects of this compound in various cancers.^[111-113] The ability of metformin to act as an anticancer drug has been attributed to both the indirect effects of lowered insulin levels and the direct targeting of tumor cells, as we already mentioned in section 2.

Recently, various studies have suggested an effect of metformin in CSCs differentiation. For example, the impact of this small molecule on gastric cancer stem cells has recently been described.^[114] The potential of metformin to influence expression of CSCs markers (CD44 and Sox2) as well as other differentiation markers such as Kruppel-like factor 4 (KLF4) and Mucin 5AC (MUC5AC) was investigated. While KLF4 is downregulated, MUC5AC is upregulated thus showing that metformin decreases the pool of CSCs in favor of differentiated cells and is able to induce the differentiation of CSCs in gastric cancer. Metformin also showed a biological activity on colorectal cancer (CRC).^[115] Indeed, Metformin was studied on two cell lines HT29 [metformin-sensitive] and SW620 [metformin-resistant] and it showed a significantly decreased proportion of CSCs among the HT29 cells. While decrease of CSC occurs in a dose-dependent manner, metformin does not affect the proportion of CSCs in SW620 cells. Metformin thus shows different effects on different CRC cell lines and affects CSCs more importantly than non-CSCs in the metformin-sensitive cells lines. The mechanism of action that constitutes the basis for these effects seems to be the influence of metformin in the glutamine pathway but further studies are needed to better understand these effects at the molecular level. In 2019, Bishnu et al. explored the effect of metformin on epithelial ovarian cancer (EOC).^[116] These authors found that metformin inhibits the acquirement of chemoresistance by inducing CSCs differentiation and maintains a more proliferative cellular state that is susceptible to chemotherapeutic intervention. Treatment of resistant cells in the presence of metformin induces a lower percentage of CSCs compared to untreated cells, further confirming the role of metformin in differentiation of cancer stem cells and in drug sensitization. This work also suggests that metformin has the ability, when combined with cisplatin-paclitaxel chemotherapy, to prevent and/or decrease development of resistance. Noteworthy these promising results cannot be extrapolated to every cancer. A recent study from Kuo et al. explained that metformin has diametrically opposed effects on head and neck squamous cell carcinoma (HNSCC).^[117] It was observed *in vitro* that metformin results in the chemoprotection of HNSCC stem cells and decreases the ability of non-stem cancer cells to proliferate. The authors suggested that metformin reduces reactive oxygen species (ROS) levels through complex III inhibition. Because high ROS levels cause CSCs differentiation or eradication, the direct effect is the elevation in the stemness of HNSCC CSCs and their maintenance after application of cisplatin.

Altogether, these studies prove the anti-CSC effect of the metformin. The combination of metformin with other drugs used in conventional chemotherapies could lead to efficient anticancer therapies, but

it has to be taken into account that this compound bears various biological effects whose some examples have been given in section 2, and the complexity of its mechanism of action has yet to be completely understood.

3.6 Curcumin

Curcumin is the principal pigment of turmeric powder and it belongs to the class of curcuminoids being responsible for the color as well as the therapeutic effects of turmeric. Widely used in Ayurvedic medicine for its anti-oxidant, antiseptic, analgesic or anti-inflammatory properties, curcumin has been consumed as a dietary supplement for centuries.^[118] It was also found in the 1990s that this molecule, also called diferuloylmethane (compound **19**, *Figure 8*), has anticancer activities since it induces cancer cells death in gastric and colon cancers, human melanoma and lung cancer by a variety of mechanisms of action, without major cytotoxic effects on healthy cells.^[119-120] First isolated in 1815, the investigations about the use of curcumin against CSCs started in 2012 on esophageal squamous cell carcinoma (ESCC) where it showed a permanent reduction in the CSC-like subpopulation.^[121] The specific mechanism causing this reduction was not examined but curcumin may force the CSCs to differentiate more frequently into non-CSCs by altering the balance between symmetrical and asymmetrical division processes. This conclusion is in agreement with the study of Zhuang et al. who observed that curcumin has low toxicity on normal cells, induces differentiation of glioblastoma cancer stem cells and efficiently inhibits growth of GSCs *in vivo* and *in vitro* by activating autophagy.^[122] Studies on stem cells from surgically resected human glioblastoma led to the observation that curcumin treatment induces an acquisition and/or an increase in differentiation's markers (Tuj1, GFAP, tubulin and Olig2) in cells treated with curcumin. Together with a decrease in the expression of neural stem/progenitor markers (CD133 and Nestin), this indicates that curcumin induces the differentiation of GSCs.^[123-124] These findings support a role for curcumin as an adjunct to traditional chemotherapy and radiation treatments in the treatment of GBM.

Curcumin, as other natural products described in this review, shows promising activity not only for the inhibition of cancer cells proliferation but also for the induction of CSCs differentiation. Even if its exact mechanism of action and applicability for this kind of biological activity has still to be completely elucidated, curcumin surely represents an interesting scaffold for chemical modification toward the improvement of its biological activity and potentially its ability to induce the differentiation of CSCs.

3.7 Other compounds inducing CSC differentiation

The small-molecule compounds described above demonstrated the ability to induce the differentiation of CSCs in various cancers and their biological activity has been largely evaluated. Some other compounds however were reported to have this promising biological activity and bear an interest in this field. As mentioned in the introduction, Wnt/ β -catenin pathway is deregulated in CSCs thus representing an attractive target for anticancer therapies. A high-throughput screening combined with ELISA assays was performed to identify small molecules able to disrupt the interaction between β -catenin and TCF4 (T-cell factor), a transcription factor essential for signal transduction. The screening of a library of 16,000 synthetic compounds led to the identification of LF3, a 4-thioureido-benzene sulfonamide derivative (compound **20**, *Figure 8*) as an efficient inhibitor of this interaction.^[125] This molecule inhibits the interaction between β -catenin and TCF4 *in vitro* and the direct consequence is the overcoming of Wnt signaling pathway deregulation. LF3 also suppresses features of cancer cells related to Wnt signaling, including high cell motility, cell-cycle progression, and the overexpression of Wnt target genes. However, LF3 does not cause cell death. Remarkably, the self-renewal capacity of cancer stem cells is blocked by LF3 in a concentration-dependent manner. Finally, LF3 reduces tumor growth and induces differentiation in a mouse xenograft model of colon cancer. The exact mechanism by which LF3 disrupts β -catenin/TCF4 interaction remains to be elucidated. The reported hypothesis is that the negatively charged sulfonamide group of LF3 may compete with Asp16 of TCF4 to bind to the positively charged pocket of β -catenin formed by Lys435. The hydrophobic tail of LF3 might insert itself into a hydrophobic cleft lined by the residues Cys466 and Pro463 of β -catenin to facilitate interaction.

An analogous screening was performed to identify small molecule antagonists of β -catenin pathway.^[126] In this screening, the authors challenged transformed colorectal cells with a secondary structure-templated chemical library including 5,000 compounds looking for compounds that inhibit a β -catenin-responsive reporter. ICG-001 (compound **21**, *Figure 8*) was identified as a specific inhibitor of the canonical Wnt signaling pathway in cancer stem cells with potential antineoplastic activity. This compound bears a tetrahydro-1*H*-pyrazino[1,2-*a*]pyrimidine-4,7(6*H*,8*H*)-dione skeleton (a β -turn bicyclic peptidomimetic) and is able to inhibit β -catenin/TCF-mediated transcription *in vitro* with micromolar efficacy. Binding studies using ICG-001 show that it selectively blocks the interaction between cyclic AMP response element binding protein (CBP) and β -catenin by binding at a minimal region at the the NH₂ terminus of CBP (amino acids 1–111) resulting in the suppression of a subset of Wnt/ β -catenin-driven gene expression. Most interestingly, ICG-001 does not interfere with p300 that is another cofactor of β -catenin. The switch from β -catenin/CBP to β -catenin/p300 controls fundamental stem and/or progenitor cell activities and CBP cofactor is known to increase self-renewal and suppresses differentiation in different types of cancers while p300 has the opposite effect.^[114] In an analogous work, it was found that ICG-001 could safely eradicate the drug-resistant CSC-like

population and initiate cell differentiation in leukemia (pre-B ALL cells).^[127] The same authors also showed that ICG-001 enforces differentiation of salivary gland tumor CSC and this approach can potentially be efficient on neck cancer in general.^[128] A second generation specific β -catenin/CBP antagonist was developed by preparing the phosphate derivative of ICG-001 thus leading to compound PRI-724 (compound **22**, *Figure 8*) approximately 20 times more potent.^[129] PRI-724 is currently in clinical trials for its use in combination with other cytotoxic compounds such as gemcitabine or cisplatin.

Another high-throughput screening of a library consisting of 108,269 compounds followed by structure-activity relationships allowed for the identification of a highly potent MELK inhibitor that inhibits the proliferation of various types of CSCs: compound OTSPS167 (or OTS167, compound **23**, *Figure 8*).^[130] MELK (Maternal embryonic leucine zipper kinase) is a member of the AMPK serine/threonine kinase family and is known to play critical roles in the formation and maintenance of cancer stem cells.^[119] MELK is also one of the markers that characterize cancer stem cells in tumors, such as breast cancer and glioblastoma. OTS167 bears a 1,5-naphthyridine core with methylketone, trans-4-((dimethylamino)methyl) cyclohexylamino and 3,5-dichloro-4-hydroxyphenyl substitutions. First, this compound showed a submicromolar inhibition activity of MELK. This activity has been linked to the suppression of the growth of human breast cancer cells and acute myeloid leukemia cells *in vitro*.^[130-132] It was also shown that this compound can affect small cell lung cancer (SCLC) and that this MELK-targeting strategy could be applied as a differentiation therapy, forcing the SCLC cells to resume the neuronal differentiation from progenitor phenotype.^[133] Another study on triple-negative breast cancer showed morphological changes in different cancer cell lines upon treatment with OTS167 suggesting a differentiation-like process occurring.^[134] Altogether, these data show the great potential of OTS167 as inducer of CSCs differentiation.

Erythropoietin-producing hepatocellular (EPH) proteins are a large family of receptors with tyrosine kinases (TKs) moieties widely considered as targets for glioblastoma.^[135] Different compounds targeting the Eph receptor have been developed in cancer research. Among them, GLPG1790 is a small molecule from Galapagos whose effects as ephrin (EPH) receptor tyrosine kinase inhibitor were first reported in 2014 for the treatment of triple negative breast cancer (TNBC).^[136] This compound showed remarkable *in vivo* efficacy in a TNBC xenograft pre-clinical model. Full tumor blockage was observed and was correlated with target (ephrin receptor tyrosine kinase) inhibition in the tumor. GLPG1790 was also evaluated against glioblastoma stem cells (GSC) for its differentiation ability since it bears good oral bioavailability and is able to cross the blood brain barrier.^[137] GLPG1790 is able to reduce stem cell renewal and the growth of glioma spheres. It also induces cell detachment from sphere cultures. These results are supported by the downregulation of a number of stem cell markers (CD44, Sox2, Oct3/4, Nestin, Stro-1, CD90 and CD105) with a GLPG1790 treatment at low doses (0.5 and 1.0 μ M) as well as an increase in classical differentiation markers (β III Tubulin/Tuj1, neurofilaments, GFAP and CD44). This small molecule regulates both the differentiation status of

GSCs and the quality of the tumor microenvironment. Even if more experiments need to be done, preliminary results from this recent study indicate that GLPG1790 sensitized cells to radiotherapy and chemotherapy.

Another small molecule playing a tumor-suppressive role in human glioblastoma (GBM) was described in 2014 and identified as CG500354 (compound **24**, *Figure 8*).^[138] The study reported by Kang et al. was conducted with three primary GBM cell cultures and demonstrated that the level of p53 expression was significantly increased upon CG500354 treatment thus inducing differentiation into neural subtypes. CG500354 targets the specific variant PDE4D (phosphodiesterase 4D) known to reduce the levels of cAMP within cells. Inhibitors of PDE4D are frequently employed to promote cAMP/CREB (cAMP response element binding protein) signaling pathway and the action of CG500354 suggests that the cAMP/CREB signaling pathway could be involved in this neural differentiation. CG500354 induces the alteration of phenotypes of human primary GBM cells from an oncogenic state into a more differentiated state.

Some other natural compounds were reported to have a differentiation activity on CSCs. For example, methylxanthine (MX) family is composed of natural compounds used in therapy for anti-inflammatory activity, induction of apoptosis, arrest of the cell cycle as well as inhibition of phosphodiesterases.^[139] Among these compounds, Theophylline (compound **25**, *Figure 8*), the 1,3-dimethyl-xanthine bioactive compound mostly present in *Camellia sinensis* (L.), was tested on melanoma.^[140] Malignant melanoma-initiating cells (MICs) showed a change in cell morphology upon treatment with theophylline, suggesting a differentiation activity. An increase of enzymatic activity in transglutaminase, that is considered as a good differentiation marker in melanoma models, was also observed showing that theophylline could represent a potential therapeutic strategy in combination with other therapeutic approaches.

Another natural compound, Morusin (compound **26**, *Figure 8*), a flavone substituted by hydroxy groups, a phenyl group and a 2,2-dimethyl pyran group, isolated from root bark of *Morus alba* might act upon targeting CSCs. This molecule showed a cytotoxic activity against human cancer cells^[141-142] and more recently growth inhibition, migration inhibition and apoptosis of human cervical CSCs.^[143] In a study focused on glioblastoma stem cells (GSCs), this compound showed the ability to suppress GSCs proliferation in a dose dependent manner *in vitro* and the reduction of the weight of tumor masses *in vivo* suggesting a growth decrease in glioblastoma initiated from GSCs. It was demonstrated that GSCs are differentiated into adipocyte-like cells at low concentrations of Morusin while apoptosis is observed at higher doses. These findings suggest the possibility of a novel strategy in cancer differentiation therapy *via* switching the identity of human cancer cells into adipocytes through transdifferentiation induction.

4. Summary and Outlook

This review summarizes the most important examples of small molecules targeting selectively CSCs in order to induce their differentiation, inhibit their tumorigenicity and sensitize them to classical chemotherapy. A number of CSCs targeting compounds that are cytotoxic selectively on these cells represent an efficient and promising strategy, but their clinical applications remain limited. The use of drugs that could target CSCs, induce their differentiation but lacking cytotoxicity and thus likely bearing reduced side effects represent a very promising strategy for tackling so far incurable cancers. The examples described here show that various compounds have the ability to induce differentiation and these have been discovered upon high-throughput screening on a particular target or studying already known drugs for other potential effects at non-cytotoxic doses. High-throughput screening search for differentiating compounds have been performed using phenotypic assays. For this reason, the exact mechanism of action at the molecular level is not always known and further studies are necessary to understand how the induction of CSCs differentiation is mediated inside cells and if this effect is specific. Similarly, drugs already employed in the clinical practice that have been discovered to induce the differentiation of CSCs, exert this effect at doses lower than the cytotoxic ones. This also suggests that the mechanism of action is probably different and needs to be investigated. However, some target-based assays begin to be developed leading to molecules directed against a specific target as well as to a better understanding of the mechanism of interaction. Only these detailed studies will allow for the improvement of biological activity based on structural studies of the interaction between the compound and the target. Noteworthy, various compounds described here are not cytotoxic at doses that induce differentiation and are meant to be applied in combination therapies with classical cytotoxic compounds likely at lower doses toward tumor eradication. These approaches are extremely promising since combination therapies hold the promise for efficient chemotherapies. It is now clear that targeting CSCs represents an important challenge for the discovery of efficient anticancer strategies since these cells are responsible for resistance to therapy and even more importantly for recurrence even when chemotherapy works on differentiated tumor cells. The differentiation approach is thus complementary to the one looking for cytotoxic agents against CSCs. This strategy is still in its infancy and the compounds presented here could be the starting point for future studies about the discovery of new molecules inducing efficient differentiation of CSCs.

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Conflict of Interest

The authors declare no conflict of interest.

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Figures

Figure 1. Chemical structure and proposed mechanism of action for some of the small molecules that have been studied as cytotoxic compounds targeting CSCs.

Figure 2. Chemical structure of all-trans retinoic acid (ATRA, **1**) together with some of its analogs (**2a-d**) and proposed mechanism of action based on the interaction with nuclear receptors RAR and RXR.

Figure 3. Chemical structure of calcitriol (**3**) and active analog BXL0124 (**4**) together with their possible mechanism of action based on the activation of VDR and RXR and their action on VDRE gene.

Figure 4. Chemical structure of Smac mimetics TL32711 (**5**), AT406 (**6**), BV6 (**7**) and their potential mechanism of action involving IAP inhibition and NF- κ B pathway activation.

Figure 5. Chemical structures of histone deacetylase inhibitors Abexinostat (**8**), MC1742 (**9**), SAHA (**10**), Valproic acid (**11**) and Trichostatin A (**12**). The inhibition of HDAC in the nucleus is illustrated as the main mechanism of action.

Figure 6. Chemical structures of IDH inhibitors Edasidenib (**13**), Ivosidenib (**14**), AG-881 (**15**) and their corresponding mechanism of action inside cells.

Figure 7. Chemical structures of DHODH inhibitors ML390 (**16**) and BAY2402234 (**17**) and illustration of their action on DHODH and following intracellular pathway.

Figure 8. Chemical structure of Metformin (**18**), Curcumin (**19**), LF3 (**20**), ICG-001 (**21**), PRI-724 (**22**), OTSPS167 (**23**), CG500354 (**24**), Theophyllin (**25**) and Morusin (**26**).

Figure 1.

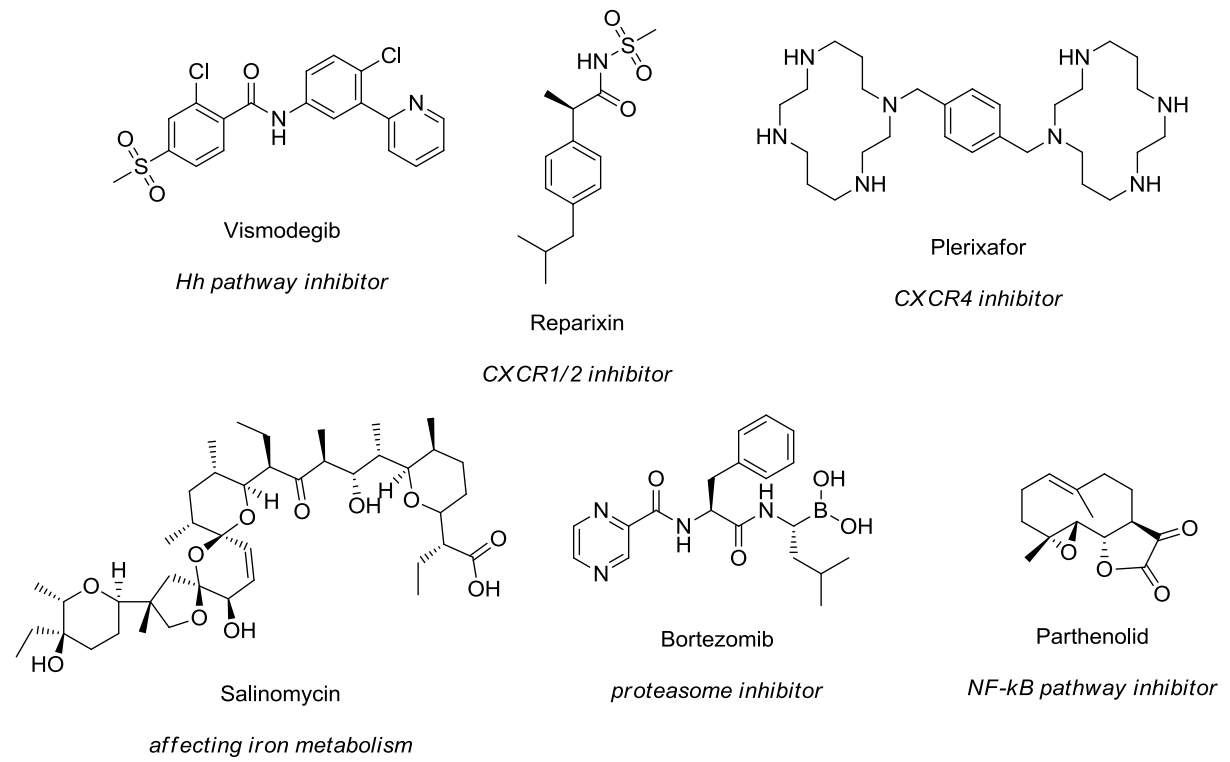


Figure 2.

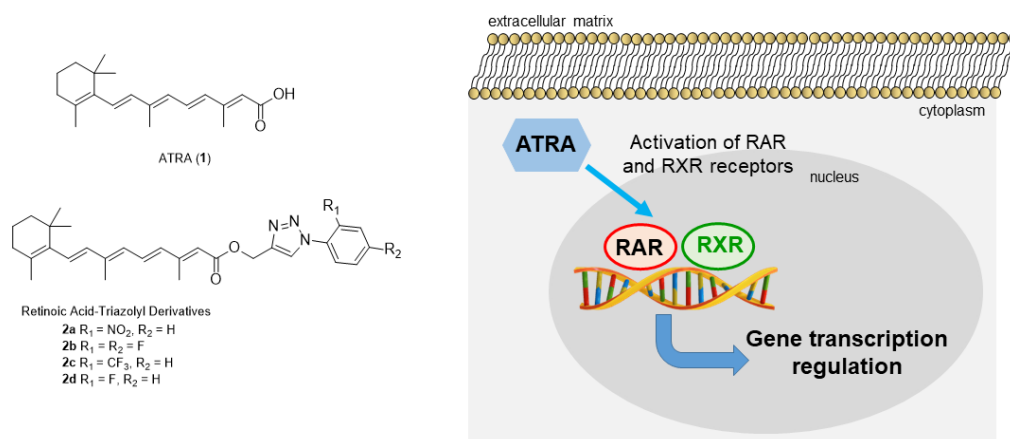


Figure 3.

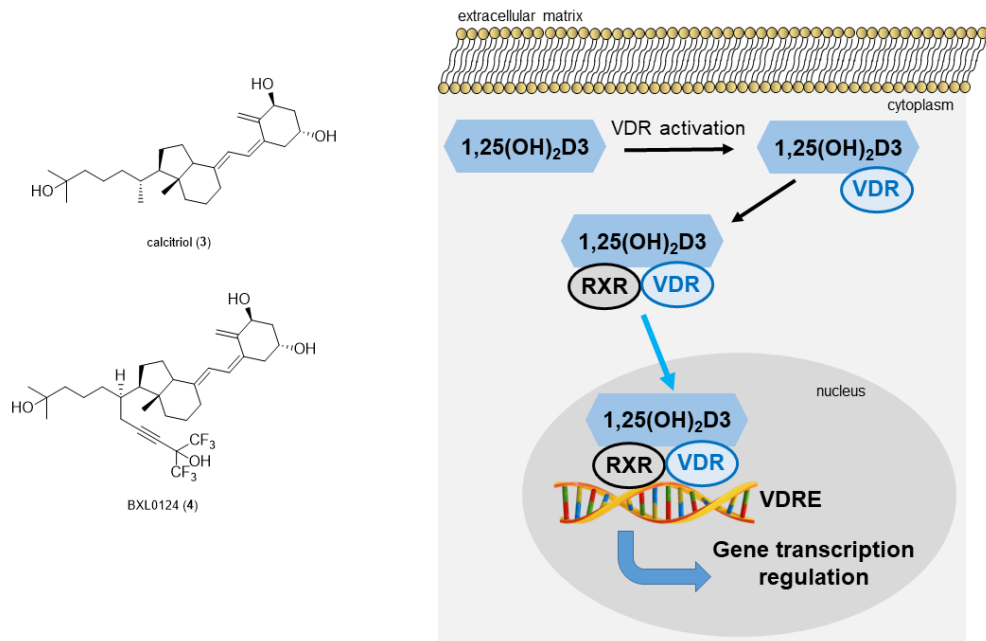


Figure 4.

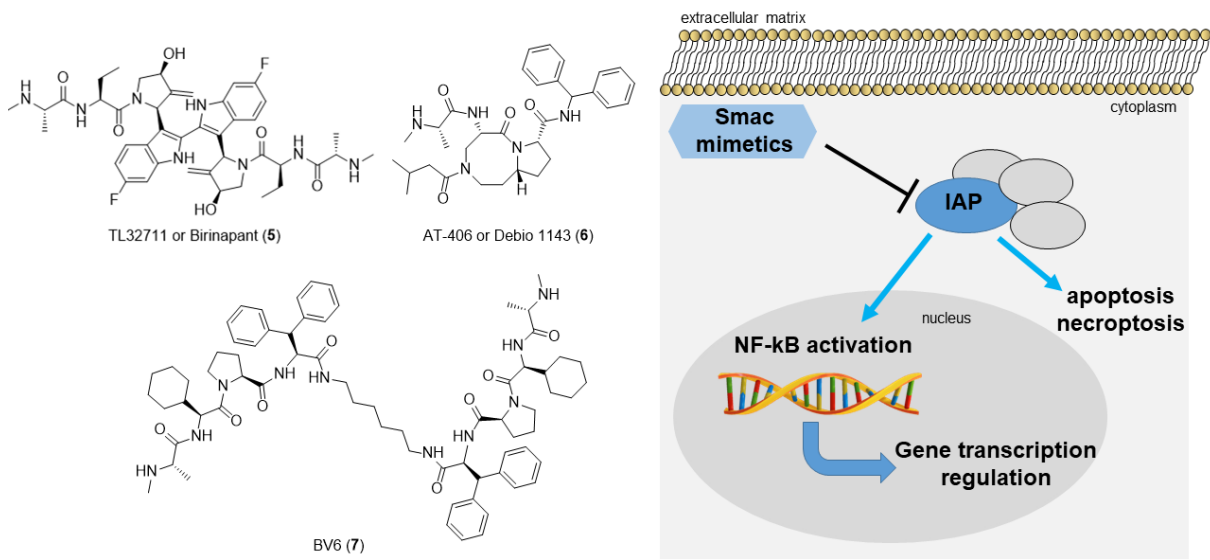


Figure 5.

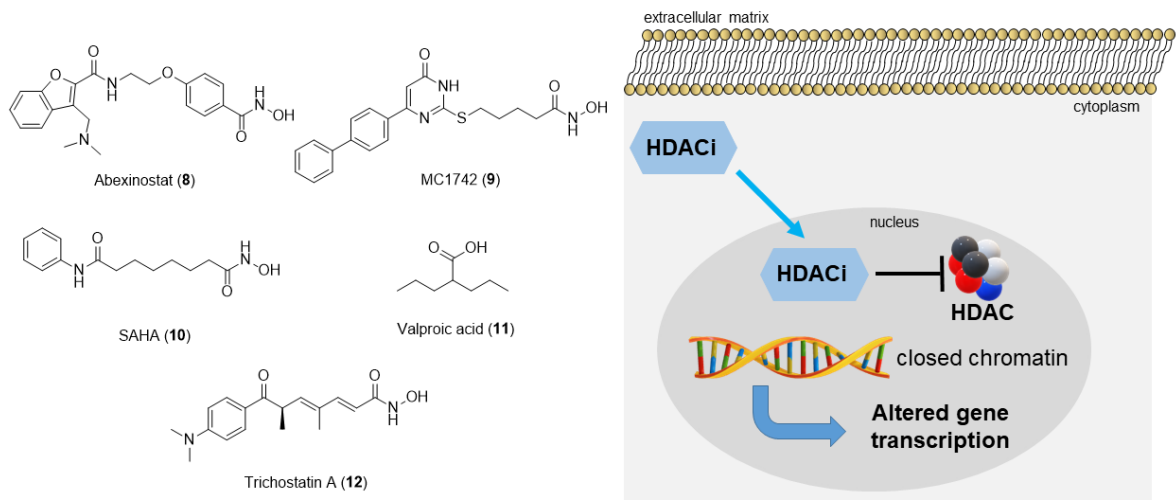


Figure 6.

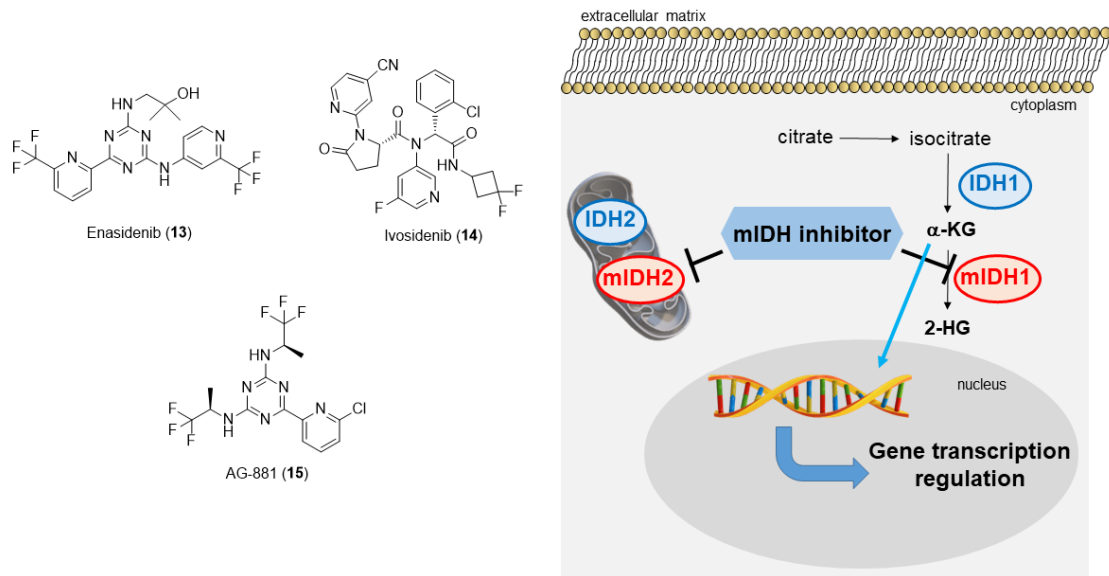


Figure 7.

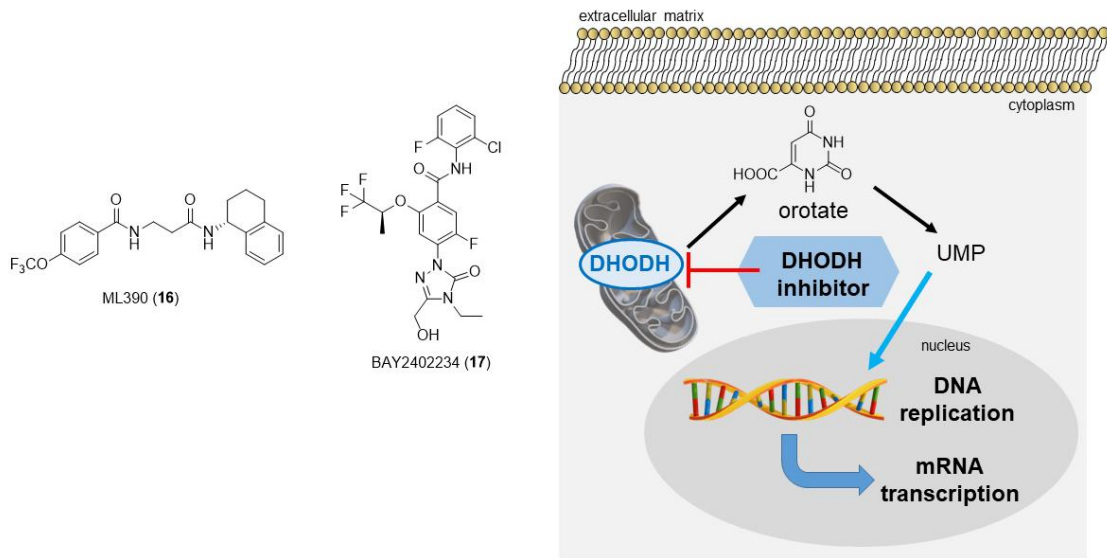


Figure 8.

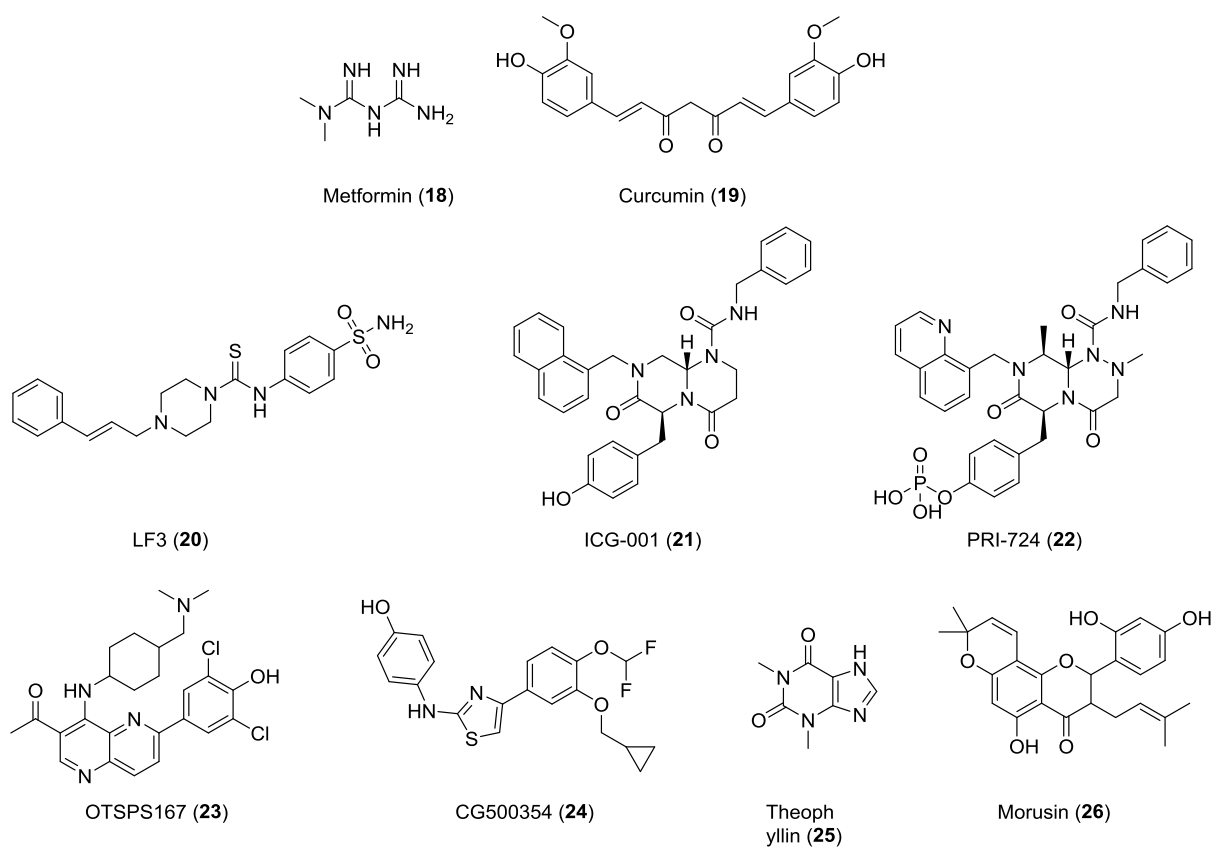


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Cancer stem cells (CSCs) are one of the main reason of therapeutic failure in a number of different tumors. In this review, we describe current examples of small-molecule drugs able to induce the differentiation of CSCs toward cancer cells that are sensitive to chemotherapy. The combination of this kind of compounds with chemotherapy could lead to tumor eradication thus representing an extremely promising approach for future therapies.

