Targeting Cancer Stem Cells with Defined Compounds and Drugs

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Abstract: Cancer stem cells (CSCs) are a subpopulation of tumor cells that possess self-renewal and tumor initiation capacity and the ability to give rise to the heterogenous lineages of cancer cells that comprise the tumor. CSCs possess numerous intrinsic mechanisms of resistance to chemotherapeutic drugs, novel tumor-targeted drugs and radiation therapy, allowing them to survive current cancer therapies and to initiate tumor recurrence and metastasis. Recently, different pathways that confer resistance and survival of CSCs, but also compounds and drugs that selectively target some of these pathways in CSCs have been identified. Such compounds and drugs include antibiotics like salinomycin, phytochemicals such as parthenolide, cyclopamine, EGCG, resveratrol, curcumin, sulforaphane and oxymatrine, the small molecule inhibitors vismodegib and repertaxin, monoclonal antibodies and antibody constructs raised against cell surface proteins expressed by CSCs, and, surprisingly, some classical drugs such as metformin, tranilast and thioridazine. These agents exhibit significant anti-CSC activity, alone or in combination with cytostatic drugs or tumor-targeted drugs, as recently shown *in vitro* and in human xenograft mice. Since current cancer therapies fail to eliminate CSCs, leading to cancer recurrence and progression, selective targeting of CSCs with compounds and drugs introduced herein may represent a novel therapeutic strategy to eradicate cancer.

Keywords: Cancer stem cells (CSCs), novel therapeutics, novel drugs, targeted therapy, combination therapy.

1. MECHANISMS OF THERAPEUTIC RESISTANCE AND SURVIVAL OF CSCs

The experimental demonstration of CSCs in a variety of human malignant tumors, including cancers of the blood, breast, brain, bone, skin, liver, lung, bladder, ovary, prostate, colon, pancreas and head and neck has led to the conceptual hypothesis that tumors, like physiologic proliferative tissues, can be hierarchically organized and propagated by limited numbers of dedicated stem cells [1-8]. According to a consensus definition, these CSCs are cells within a tumor that possess the capacity to self-renew and to give rise to the heterogeneous lineages of cancer cells that comprise the tumor [9]. CSCs can be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor in serial xenotransplantation settings [9], and recent studies provide evidence for the existence and relevance of CSCs in clinical therapeutic situations [10-12].

Unfortunately, CSCs possess numerous intrinsic mechanisms of resistance to conventional chemotherapeutic drugs and radiation therapy, including expression of ATP-binding cassette (ABC) drug pumps, such as ABCG2/BCRP and P-glycoprotein/MDR1 [13-17], activation of Wnt/β-catenin signaling [18-21], activation of the Hedgehog and Notch signaling pathways [20-25], expression and activation of the Akt/PKB and ATR/CHK1 survival pathways [26-28], aberrant PI3K/Akt/mTOR-mediated signaling and loss of phosphatase and tensin homolog (PTEN) [29-31], amplified activity of aldehyde dehydrogenase 1 (ALDH1) [32-35], amplified checkpoint activation and efficient DNA and oxydative damage repair [36-42], acquisition of epithelial-mesenchymal transition (EMT) [10, 24, 43, 44], constitutive activation of NF-κB [45-47], expression of CD133/prominin-1 and general radioresistance [36, 37, 48-50], radiation-induced conversion of cancer cells to CSCs [51], protection from apoptosis by autocrine production of interleukin-4 [52, 53], various mechanisms of apoptosis resistance and defective apoptotic signaling [49, 54, 55], protection by microenvironment and niche networks [29, 56, 57], metabolic alterations with a preference for hypoxia [58, 59], immune evasion [60-62], low proliferative activity [63], and, ultimately, transient or long-termed quiescence, the latter also termed dormancy [64, 65].

Many of these intrinsic mechanisms as well as yet unknown mechanisms of resistance and immortality [66-69] allow CSCs to survive current cancer therapies and to initiate reconstitution of the original tumor, long-term tumor recurrence and metastasis [10-12, 70-75].

2. RESISTANCE OF CSCs TO NOVEL TUMOR-TARGETED DRUGS

Similar to conventional anticancer drugs, numerous novel tumor-targeted drugs were designed to target

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rapidly proliferating cancer cells, so that CSCs might be relatively insensitive to these drugs. For instance, imatinib has been developed as an inhibitor of the Bcr-Abl tyrosine kinase, which constitutes the fusion protein product of a chromosomal translocation (so called Philadelphia chromosome) that acts as a molecular switch for proliferation and differentiation of multipotent progenitor cells in chronic myeloid leukemia (CML) [76]. Imatinib has been shown to eliminate proliferating, committed leukemia progenitors, but not nonproliferating CML stem cells [77-79], which persist after imatinib therapy [80], and, after initial therapeutic success, many CML patients become resistant to imatinib therapy [81].

Furthermore, trastuzumab, a humanized monoclonal antibody directed against HER2 has been developed to treat patients with HER2-overexpressing breast cancers that represent one fourth of all breast cancer patients [82]. HER2 is a member of the human epidermal growth factor receptor tyrosine kinase family that is preferentially expressed in breast and ovarian cancer and that activates signaling pathways that promote tumorigenic cellular processes, such as proliferation and evasion of apoptosis [83, 84]. However, trastuzumab mono-therapy in patients with HER2-overexpressing metastatic breast cancer shows a response rate of no more than 30 % [85], and primary or acquired resistance to trastuzumab occurs frequently in different clinical settings even when combination regimes are used [82, 86, 87]. One important mechanism of trastuzumab resistance in the therapy of HER2-overexpressing breast cancer might be the failure of trastuzumab to target breast CSCs [73, 88-90]. Trastuzumab has been shown to exert its antitumor activities only effectively in the presence of a normal PI3K signaling pathway and in the presence of PTEN [91-94], but, as noted above, CSCs display aberrant PIK3K signaling and loss of PTEN [29-31]. A similar scenario has been reported for the small molecule dual inhibitor of EGFR tyrosine kinase and HER2 tyrosine kinase, lapatinib, which loses its therapeutic activity in breast cancers displaying loss of PTEN [95].

Finally, sorafenib, a small molecule inhibitor of multiple tyrosine kinases involved in tumor proliferation and tumor angiogenesis, including Raf, VEGFR, PDGFR and FLT3 [96] has been established in the treatment of acute myeloid leukemia (AML) because FMS-like tyrosine kinase 3 (FLT3) is overexpressed in leukemic blasts in almost all cases of AML [97]. As demonstrated recently, sorafenib is capable of reducing the number of mature AML progenitors, but fails to eradicate AML stem and primitive progenitor cells due to robust protection of these cells by the bone marrow stromal microenvironment [98], providing a further paradigm that novel tumor-targeted drugs fail to eliminate CSCs.

3. THE CHALLENGE OF TARGETING CSCs AND THEIR PROGENY

According to the cancer stem cell concept of carcinogenesis [1, 3, 4, 7-9, 99], CSCs represent novel and translationally relevant targets for cancer therapy, and the identification, development and therapeutic use of compounds and drugs that selectively target CSCs is a major challenge for future cancer treatment [5, 67, 100-102]. However, the goal for any CSC-directed therapy should be the eradication of all CSCs in a patient, and the efficacy of single agents targeting CSCs may be limited by several factors. CSCs can represent a heterogenous population that may not be homogeneously sensitive to a given anti-CSC agent [8, 103-105], and, under the selection pressure of agents targeting CSCs, therapy resistant CSC clones may emerge [106]. Therefore, the eradication of all CSCs will likely require targeting of more than one intrinsic pathway operating in CSCs to reduce the probability of escape mutants [100, 107-109]. Moreover, agents causing tumor regression in advanced stages of cancer likely reflect effects on the bulk tumor population, but may have minimal effect on the CSC population. In contrast, a CSC-specific therapy would show modest effect on tumor growth of the bulk tumor population in advanced stages of cancer, but may have substantial clinical benefit in early stages of cancer as well as in neoadjuvant and adjuvant clinical settings [101]. Ultimately, cure of cancer will require the eradication of all malignant cells within a patient´s cancer: CSCs and their progeny. Therefore it will be important and promising to combine in sophisticated clinical settings CSC targeting agents with novel tumor-targeted drugs and conventional cytotoxic drugs. Such combinations may act in concert to eradicate CSCs, more differentiated progenitors and bulk tumor cells in cancer patients [110-117].

4. COMPOUNDS AND DRUGS THAT TARGET CSCs

Various compounds and drugs that selectively target CSCs have been discovered recently [67, 100, 102, 118], (Table **1**). These agents include microbial-derived and plant-derived biomolecules [119-123], small molecule inhibitors targeting key components of

intrinsic signaling pathways of CSCs [31, 124-126], antibodies directed against CSC-specific cell surface molecules [127-129], and, surprisingly, some classical drugs, such as metformin [130-133], tranilast [102, 134] and thioridazine [135] that have been used for decades for the treatment of metabolic, allergic, and psychotic diseases, respectively.

Although these compounds and drugs have been shown to effectively target signaling pathways and/or molecules selectively operating in CSCs, some of them are also capable of killing other types and subpopulations of cancer cells, which do not display CSC properties. In particular, the biomolecules salinomycin and parthenolide as well as the biguanide metformin have been demonstrated to induce apoptosis in various types of human cancer cells [120, 136, 137], suggesting that these compounds may contribute to the eradication of cancer more effectively than compounds targeting either CSCs or regular cancer cells. Moreover, the ionophore antibiotic salinomycin seems to have even extended capabilities of eliminating cancer, because this compound has been demonstrated to effectively target regular cancer cells [16, 138-140], highly multidrug and apoptosis resistant cancer cells [16, 138, 141], and CSCs [16, 116, 117, 141-146]. Finally, recent data obtained *in vitro* and in xenograft mice bearing human cancers indicate that CSC targeting agents are most effective in eradicating CSCs and their progeny when these agents are combined with conventional cytostatic drugs and/or novel tumor-targeted drugs [90, 116, 117, 131, 141, 143, 147-152], envisioning the use of complex combination therapies for the future treatment of cancer.

4.1. Microbial-Derived Compounds/Antibiotics

4.1.1. Salinomycin

Salinomycin is a monocarboxylic polyether antibiotic that was originally isolated from the culture broth of the actinobacterium *Streptomyces albus* (strain No. 80614) [153]. The large 751 Da pentacylic molecule with a unique tricyclic spiroketal ring system and an unsaturated six-membered ring constitutes a lipophilic, anionic and weakly acidic compound with the molecular formula $C_{42}H_{70}O_{11}$ [153, 154]. Salinomycin acts in biological membranes, including cytoplasmic and mitochondrial membranes, as a monovalent cation ionophore with strict selectivety for alkali ions and a strong preference for K^+ [155], thereby promoting mitochondrial and cellular K^+ efflux and inhibiting mitochondrial oxidative phosphorylation [156].

Salinomycin displays antimicrobial activity against Gram-positive bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus flavus*, *Sarcina lutea* and *Mycobacterium spp*., some filamentous fungi, *Plasmodium falciparum* and *Eimeria spp*., the latter constitute protozoan parasites responsible for the poultry disease coccidiosis [153, 157, 158]. Therefore, salinomycin is used until today as an anticoccidial drug in poultry and is also fed to ruminants and pigs to improve nutrient absorption and feed efficiency [157, 159]. Furthermore, salinomycin is a positive ionotropic and chronotropic agent that increases cardiac output, left ventricular systolic pressure, heart rate, mean arterial pressure, coronary artery vasodilatation and blood flow, and plasma catecholamine concentration, as demonstrated in mongrel dogs receiving a single intravenous injection of 150 $\mu g \bullet kg^{-1}$ salinomycin [160]. For several reasons, salinomycin has never been established as a drug in humans until now. For instance, a case of an accidental inhalation and swallowing of about 1 $mg \bullet kg^{-1}$ salinomycin by a 35-year-old male human revealed severe acute and chronic salinomycin toxicity with acute nausea, photophobia, leg weakness, tachycardia and blood pressure elevation, and a chronic creatine kinase elevation, myoglobinuria, limb weakness, muscle pain and mild rhabdomyolysis [161]. Risk assessment data recently published by the European Food Safety Authority declare an acceptable daily intake (ADI) of 5 $\mu g \bullet kg^{-1}$ salinomycin for humans, and daily intake of more than 500 $\mu g \bullet kg^{-1}$ salinomycin by dogs leads to neurotoxic effects, such as myelin loss and axonal degeneration [162]. In view of this considerable toxicity in mammals, salinomycin has only been used for more than 30 years as a coccidostat and growth promoter in livestock and was not considered as a drug for humans.

It was a great surprise when Lander, Weinberg and colleagues showed in 2009 that salinomycin selectively kills human breast CSCs [142]. In a sophisticated experimental approach, the authors used oncogenic transformed immortalized human mammary epithelial cells (termed HMLER), in which knockdown of E-cadherin by RNA interference resulted in the generation of cells undergoing epithelial-mesenchymal transition (EMT), a latent embryonic program that can endow cancer cells with migratory, invasive, self-renewal and drug resistance capabilities [163-165]. These human breast cancer stem-like cells (termed HMLER-shEcad) displayed characteristic properties of CSCs, were capable of forming tumorspheres in

Table 1: Compounds and Drugs that Target CSCs

Class	Compound	Targets	Clinical Status	References
Microbial-derived, ionophore antibiotic	Salinomycin	Breast CSCs, AML SCs, gastrointestinal stroma tumor (GIST) CSCs, gastric CSCs, lung CSCs, osteosarcoma CSCs, colorectal CSCs, squamous cell carcinoma CSCs, prostate CSCs pancreatic CSCs	Phase I/II: triple negative breast cancer	[16] [116] [117] [138] [141] [142] [143] [144] [145] [146] [147] [169] [170] [171]
Microbial-derived, antibiotic	3-O-methylfunicone (OMF)	Breast CSCs	Preclinical	[188]
Plant-derived, sesquiterpene lactone	Parthenolide and dimethylamino-parthenolide LC-1	AML SCs, lymphoid leukemia SCs breast CSCs, prostate CSCs, myeloma CSCs	Phase I: LC-1, acute myeloid leukemia	[46] [197] [198] [199] [200] [201] [202] [203] [204]
Plant-derived, steroidal alkaloid (Smo antagonist, Hedgehog pathway inhibitor)	Cyclopamine and IPI-926 (Saridegib)	Glioblastoma CSCs, multiple myeloma CSCs, chronic myeloid leukemia SCs, gastric CSCs, hepatoma CSCs, breast CSCs, prostate CSCs	Phase I: IPI-926 (Saridegib), advanced solid tumors	[22] [44] [211] [212] [213] [214] [215] [216] [217] [219]
Plant-derived, catechin/polyphenol	EGCG (epigallocatechin-3-gallate) and synthetic EGCG analogs	Prostate CSCs, pancreatic CSCs, breast CSCs	Phase I-II, metastatic prostate cancer, advanced solid tumors, small cell lung cancer, various chemopreventive studies	[225] [226] [228] [229] [230]
Plant-derived, stilbenoid/ natural polyphenol	Resveratrol	Medulloblastoma CSCs, breast CSCs, pancreatic CSCs, glioblastoma CSCs	Phase I, II, colon cancer	[235] [236] [237] [238] [239] [244]

(Table 1). Continued.

Class	Compound	Targets	Clinical Status	References
Plant-derived, curcuminoid/ natural polyphenol	Curcumin, analogs GO-Y030, difluorinated-curcumin (CDF)	Glioblastoma CSCs, colon CSCs, pancreatic CSCs, breast CSCs	Phase II, advanced pancreatic and breast cancer. Phase IIa, prevention of colon cancer.	[150] [247] [251] [252] [253] [254] [256] [258] [259] [260]
Plant-derived, natural isothiocyanate	Sulforaphane	Pancreatic CSCs, breast CSCs, prostate CSCs, chronic myeloid leukemia SCs	Preclinical	[151] [152] [262] [263] [266] [267]
Plant-derived, quinolizidine alkaloid	Oxymatrine	Breast CSCs	Preclinical	[264] [265]
Small-molecule inhibitor (Smo antagonist, Hedgehog pathway inhibitor)	Vismodegib (GDC-0449)	Pancretic CSCs, lung CSCs,	Phase I, II, medulloblastoma, basal cell carcinoma, glioblastoma, chondrosarcoma, colon, lung, ovarian, pancreatic, breast and gastric carcinoma.	[277] [278] [279] [280] [281]
Small-molecule inhibitor (inhibitor of CXCR1 and CXCR2)	Repertaxin	Breast CSCs	Preclinical	[124]
Classical drug (biguanide); anti-diabetic drug	Metformin	Breast CSC, pancreatic CSC, thyroid CSC	Phase I-III, breast cancer, prostate cancer, various solid tumors. Combination with conventional anti-cancer drugs.	[90] [112] [115] [130] [131] [303] [304] [305] [306] [307] [308]
Classical drug (synthetic cinnamoyl anthranilate); anti-allergic and anti-fibrotic drug	Tranilast	Breast CSCs	Preclinical	[134]
Classical drug (piperidine phenothiazine); neuroleptic-/antipsychotic-drug	Thioridazine	AML SCs	Preclinical	[135]
Monoclonal antibody	H90 (anti-CD44)	AML SCs	Preclinical	[332]

(Table 1). Continued.

Class	Compound	Targets	Clinical Status	References
Monoclonal antibody	P245 (anti-CD44)	Breast CSCs	Preclinical	[333]
Monoclonal antibody	B6H12.2 (anti-CD47)	AML SCs, bladder CSCs	Preclinical	[342] [343]
Monoclonal antibody	7G3, CSL362 (anti-CD123)	AML SCs	Phase I: CSL362, AML	[347]
Bispecific antibody, bifunctional	MT110 (anti-EpCAM/ anti-CD3)	Colon CSCs, pancreatic CSCs	Phase I, advanced solid tumors	[352] [353]
Bispecific antibody, trifunctional	Catumaxomab (anti-EpCAM/ anti-CD3)	CSCs in malignant ascites induced by human ovarian, gastric and pancreatic cancer	Phase I-III, malignant pleural effusions, malignant ascites, peritoneal carcinomatosis, non-small cell lung cancer, ovarian, gastric and epithelial cancer	[357] [358] [359] [360]

suspension cultures (a standard clonogenic assay for the detection of self-renewal of CSC, Ref. 9), showed high and low expression of CD44 and CD24, respectively, and exhibited resistance to chemotherapeutic drugs and cytotoxic agents, such as paclitaxel, doxorubicin, actinomycin D, camptothecin and staurosporine [142]. In a high-throughput screening approach, about 16,000 compounds from chemical libraries, including biological molecules and natural extracts with known bioactivity, were tested for activity and toxicity against HMLER-shEcad cells. From a pool of 32 promising candidates, only one compound markedly and selectively reduced the viability of breast cancer stem-like HMLER-shEcad cells: salinomycin. It was further demonstrated that salinomycin, in contrast to the anti-breast cancer drug paclitaxel, selectively reduces the proportion of $CD44^{high}/CD24^{low}$ CSCs in cultures of mixed populations of HMLER-shEcad cells and control cells that had not undergone EMT. In addition, pre-treatment of HMLER-shEcad cells with salinomycin resulted in inhibition of HMLER-shEcad-induced tumorsphere formation, which was not observed after pre-treatment of the cells with paclitaxel [142]. Using comparative global gene expression profiling, it was shown that, in $CD44^{high}/CD24^{low}$ HMLER cells, salinomycin, but not paclitaxel, was capable of changing gene expression signatures characteristic of breast CSCs or mammary epithelial progenitor cells isolated from human breast cancers. In particular, expression of genes that inversely correlates with metastasis-free survival, overall survival and clinical outcome of breast cancer patients [166, 167], was down-regulated by salinomycin. Expression of a set of genes that promote the expansion of mammary epithelial stem cells and the formation of tumorspheres [168] was also markedly down-regulated by salinomycin, but not by paclitaxel. In contrast, genes involved in mammary epithelial differentiation that encode membrane-associated and secreted proteins of the extracellular matrix were up-regulated by salinomycin [142]. Finally, as a proof of principle, it was demonstrated that salinomycin inhibits the ability of breast CSCs to form tumors in mice. Pre-treatment of HMLER cells for 7 days with salinomycin and subsequent serial limiting dilution and injection of the cells into NOD/SCID mice resulted in a >100-fold decrease in tumor-seeding ability, relative to pretreatment of the cells with paclitaxel. Finally, salinomycin treatment of NOD/SCID mice with human breast cancers (xenograft mice) resulted in a reduction of the tumor mass and metastasis, and explanted tumors showed a reduced number of breast CSCs as well as an increased epithelial differentiation [142].

According to the primary finding that salinomycin induces massive apoptosis in human cancer cells that display different mechanisms of drug and apoptosis resistance [138], a subsequent study demonstrated that salinomycin is able to overcome ABC transporter-mediated multidrug resistance and apoptosis

resistance in human acute myeloid leukemia stem cells (AML SCs) [16]. As demonstrated in the study, expression of functional ABC transporters, such as P-glycoprotein/MDR1, ABCG2/BCRP and ABCC11/MRP8 in human KG-1a AML SCs confers resistance of the cells to various chemotherapeutic drugs, including cytosine arabinoside, doxorubicin, gemcitabine, 5-fluorouracil, topotecan, etoposide and bortezomib, but not to salinomycin, which was capable of inducing massive apoptosis in KG-1a AML SCs [16]. Of note, salinomycin did not permit long-term adaptation and development of resistance of KG-1a AML SCs to apoptosis-inducing concentrations of salinomycin, whereas the cells could readily be adapted to survive and to proliferate in the presence of initially apoptosis-inducing concentrations of doxorubicin and bortezomib [16], (Figure **1**).

These findings strongly suggest that salinomycin is capable of targeting breast CSCs and AML SCs, and a series of recent studies show similar effects of salinomycin in other types of CSCs. In gastrointestinal stromal tumors (GIST), a subpopulation of cells expressing CD44, CD34 and kit (activating stem cell factor receptor) have been identified as cells with self-renewal and tumorigenic capabilities [143]. These Kit^{low} CD44+ CD34+ CSCs are resistant to inhibition of proliferation by imatinib, a tyrosine kinase inhibitor targeting oncogenic kit signaling that is commonly used in the treatment of metastatic GIST [143]. By contrast, salinomycin nearly completely inhibited the proliferation of Kit^{low} CD44+ CD34+ CSCs without causing apoptosis, and salinomycin also promoted fibroblast-like differentiation of the cells [143]. However, a combined treatment of the cells with imatinib and salinomycin caused a significantly greater inhibition of proliferation than salinomycin alone [143]. Thus, the study demonstrates that salinomycin is able to inhibit proliferation and to induce differentiation of GIST CSCs, and also suggests that a combination of salinomycin and imatinib may provide therapeutic benefit for patients with GIST.

Similar results were obtained in CD44+ CD24- ALDH1+ breast CSCs isolated from the human breast cancer cell line MCF-7. In CD44+ CD24- ALDH1+ MCF-7-derived breast CSCs, salinomycin was capable of markedly reducing the tumorsphere formation of the cells and the percentage of ALDH1+ expressing cells by nearly 50 fold [141]. Treatment of the cells with salinomycin as well as combined treatment with doxorubicin and salinomycin, but not treatment with doxorubicin alone, reduced the cloning efficiency by 10-30 fold and markedly increased apoptosis in CD44+ CD24- ALDH1+ breast CSCs [141]. Similar results were obtained recently in MCF-7-derived breast CSCs and HER2-expressing breast cancer cells that were more effectively killed by the combination of salinomycin and trastuzumab than by salinomycin or trastuzumab alone [147], providing further evidence that salinomycin alone and particularly in combination with conventional anti-cancer drugs effectively targets CSCs.

Salinomycin has recently been shown to target CSCs in different types of human cancers, including gastric cancer [146], lung adenocarcinoma [145], osteosarcoma [169], colorectal cancer [144], squamous cell carcinoma (SCC) [170] and prostate CSCs [171], suggesting that salinomycin may be effective in targeting CSCs of many, if not all, types of human cancers, although it is currently not known whether all cancers contain subpopulations of CSCs. In ALDH1+ gastric CSCs, which displayed resistance to the conventional chemotherapeutic drugs 5-fluorouracil and cisplatin, salinomycin effectively inhibited tumorsphere formation, proliferation and viability of the cells [146]. Similar results were obtained in ALDH1+ CSCs derived form lung adenocarcinoma cells [145] and in osteosarcoma CSCs [169]. In colorectal cancer cells and in SCC cells, salinomycin, but not oxaliplatin or cisplatin, was capable of significantly reducing the proportion of CSCs, as detected in tumorsphere assays [144, 170] and in human SCC xenograft mice [170].

Importantly, salinomycin in combination with a conventional cytotoxic drug eradicates human cancers in xenograft mice much more efficiently than the single agent [116, 117]. In particular, salinomycin inhibited the growth of CD133+ pancreatic CSCs in tumorsphere formation assays, while the cytotoxic drug gemcitabine, a nucleoside analog commonly used in the treatment of metastatic pancreatic cancer, induced marked apoptosis in non-CSC CD133- pancreatic cancer cells [116]. Consistently, salinomycin combined with gemcitabine eradicated human pancreatic cancer in xenograft mice much more efficiently than either salinomycin or gemcitabine [116]. Similar results were obtained in a study using CD44+ CD24- breast CSCs sorted from the human breast cancer cell line MCF-7 [117]. Salinomycin more efficiently inhibited the proliferation of CD44+ CD24- breast CSCs than of the parental MCF-7 cells, and salinomycin was able to induce significant tumor regression and to reduce the number of CD44+ CD24- breast CSCs in tumors established by MCF-7 cells in xenograft mice [117]. Of

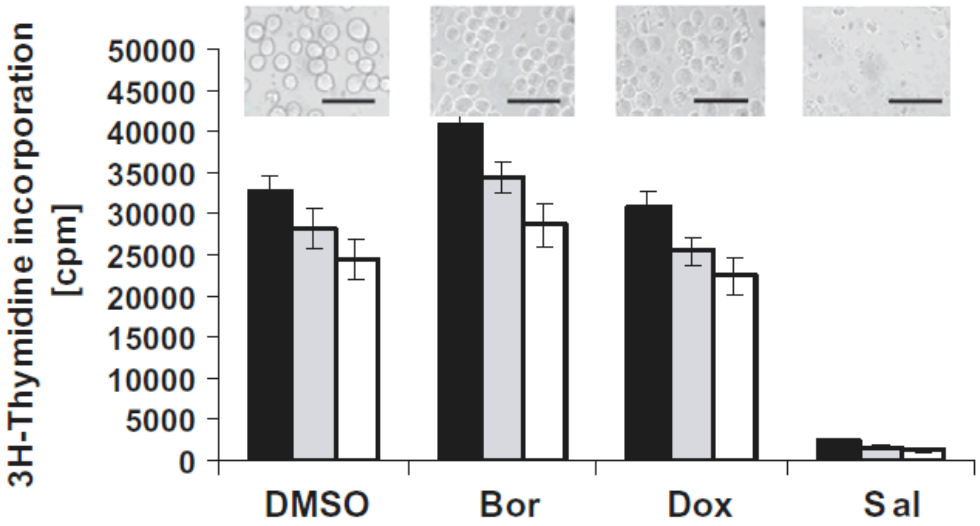

Figure 1: Salinomycin does not permit long-term adaptation and development of resistance of KG-1a AML SCs to apoptosis-inducing concentrations of salinomycin (Sal, 10 μM), whereas the cells could be readily adapted to survive and to proliferate in the presence of initially apoptosis-inducing concentrations of doxorubicin (Dox, 0.5 μg/ml) and bortezomib (Bor, 12.5nM). After 12 weeks of culturing in the presence of 12.5 nM Bor, 0.5 μg/ml Dox, 10 μM Sal or DMSO 5% (v/v), proliferation of the cells was determined by [^{3}H] thymidine incorporation for 24 h. White bars: KG-1a AML SCs; grey bars: KG-1a AML cells; black bars: KG-1 AML cells. Inserts show invert microscopic pictures (400x) of KG-1a AML SCs cultured for 12 weeks in the presence of the drugs noted below. Size bars are 50 μm.

Adapted from [16], with permission from Elsevier B.V.

note, salinomycin in combination with paclitaxel almost completely eradicated the MCF-7 tumors in xenograft mice [117]. As in the case of breast CSCs and GIST CSCs [117, 141, 143, 147], salinomycin is able to enhance in regular cancer cells the cytotoxic effects of conventional cancer drugs, such as doxorubicin, etoposide, paclitaxel, docetaxel, vinblastin and trastuzumab [147-149], envisioning a central role for salinomycin-based combination therapies in the future treatment of cancer [121].

Although the exact mechanisms underlying the elimination of CSCs by salinomycin remain poorly understood, recent work has contributed to an increased understanding of some modes of action of salinomycin in CSCs and cancer cells. It has been shown that salinomycin induces apoptosis in CSCs of different origin [16, 116, 141, 169], but the particular mechanisms of apoptosis induction by salinomycin in CSCs remain unclear and may differ among the cell type, as demonstrated for regular cancer cells [138, 140, 148, 149]. It is also evident that salinomycin is refractory to the action of ABC transporters since salinomycin is able to overcome ABC transporter-mediated multidrug resistance in AML SCs [16]. Moreover, salinomycin has been demonstrated to be a potent inhibitor of the ABC transporter P-glycoprotein/MDR1 in different cancer cells [172, 173]. Next, salinomycin has been shown to inhibit in chronic lymphocytic leukemia cells proximal Wnt signaling by reducing the levels of the Wnt co-receptor LRP6 and by down-regulating the expression of the Wnt target genes *LEF1*, *cyclin D1* and *fibronectin*, finally leading to apoptosis [139]. Most cancer cells rely more on aerobic glycolysis than on oxidative phosphorylation (the Warburg effect, Ref. 174), but, for instance, malignant transformation of human mesenchymal stem cells is linked to an increase of oxidative phosphorylation [175], and glioma CSCs have been shown to mainly rely on oxidative phosphorylation [176, 177]. In this context, salinomycin has been shown to inhibit oxidative phosphorylation in mitochondria [156] that may contribute to the elimination of CSCs by salinomycin. Salinomycin is a K^+ ionophore that interferes with transmembrane K^+ potential and promotes the efflux of K^+ from mitochondria and cytoplasm [155, 156]. Expression of K^+ channels has been documented in CD34+/CD38- AML SCs and in CD133+ neuroblastoma CSCs, but not in their non-tumorigenic counterparts [178, 179]. Moreover, a decrease in intracellular K^+ concentration by pharmacological induction of K^+ efflux is directly linked to the induction of apoptosis in cancer cells [180, 181], suggesting that mitochondrial and cytoplasmic K^+ efflux induced by salinomycin leads to apoptosis in CSCs. Finally, salinomycin is able to promote differentiation of CSCs and to induce epithelial reprogramming of cells that had undergone EMT [142, 143]. This is in concert

with the finding that salinomycin up-regulates the expression of genes involved in mammary epithelial differentiation [142]. Thus, salinomycin might target and eliminate CSCs by multiple mechanisms of which only a few are currently known. Future research may uncover an increasing number of relevant mechanisms of targeting CSCs by salinomycin.

4.1.2. 3-O-Methylfunicone

3-O-methylfunicone (OMF) is a secondary metabolite produced by the soil fungus *Penicillium pinophilum* [182] and by an Australian sea salt fungus [183]. The compound is a fungitoxic pyrone that inhibits the growth of various phytopathogenic fungi and the activities of mammalian Y-family DNA polymerases [182, 183]. OMF has been shown to induce cell cycle arrest and apoptosis in human melanoma and cervical cancer cells [184, 185] and to affect proliferation and motility of breast cancer cells that is accompanied by down-regulation of αvβ5 integrin and matrix metalloproteinase-9 and by inhibition of survivin and human telomerase reverse transcriptase (hTERT) gene expression [186]. Similar results have been obtained in human mesothelioma cells exposed to OMF or to OMF combined with cisplatin [187].

Recently, OMF has been shown to selectively deplete breast CSCs present in the human breast cancer cell line MCF-7 [188]. It was demonstrated that OMF treatment of MCF-7-derived tumorspheres markedly reduced the number and size of the tumorspheres, and pretreatment of MCF-7 cells with OMF abrogated the ability of the cells to form tumorspheres. In contrast to cisplatin, OMF was capable of eliminating breast CSCs from the MCF-7-derived tumorspheres *via* induction of apoptosis that resulted in the complete disappearance of breast CSCs expressing the stemness markers CD24, CD29 CD44, CD133 and CD338. Finally, OMF treatment of MCF-7-derived tumorsphere cells resulted in the down-regulation of *survivin*, *hTERT* and *Nanog* gene expression, pointing out that OMF affects expression of genes critical for maintenance and survival of CSCs [188].

4.2. Plant-Derived Compounds/Phytochemicals

4.2.1. Parthenolide

Parthenolide is a nucleophilic sequiterpene lactone isolated from the flowerheads and leaves of feverfew (*Tanacetum parthenium*) [189]. Partenolide inhibits the activation and nuclear translocation of transcription factor NF-κB by directly binding to IκB kinase, thereby preventing IκB kinase-mediated phosphorylation and subsequent proteasomal degradation of the NF-κB inhibitor IκB [190, 191]. Because NF-κB is a master transcription factor for inflammatory gene expression and an important player in the development, maintenance and progression of cancer [192-194], it is obvious that parthenolide exhibits significant anti-inflammatory and anti-cancer activities [195, 196].

Of note, parthenolide was the first compound discovered to selectively target human CSCs. In a seminal study, Guzman, Jordan and coworkers demonstrated that parthenolide induces apoptosis in human CD34+ CD38- AML SCs, but not in normal CD34+ hematopoietic stem and progenitor cells [197]. By contrast, cytosine arabinoside, the standard drug used for AML therapy, failed to induce significant apoptosis in AML SCs. Parthenolide-induced apoptosis of AML SCs is associated with inhibition of NF-κB activity, proapoptotic activation of tumor suppressor protein p53 and generation of reactive oxygen species [197]. In fuctional terms, parthenolide strongly reduces the ability of AML SCs to engraft in NOD/SCID mice but does not affect the activity of normal hematopoietic stem and progenitor cells to differentiate into myeloid or lymphoid lineages [197]. Similar results were obtained using the orally bioavailable parthenolide analog dimethylamino-parthenolide [198]. Not only AML SCs, but also human prostate CSCs, myeloma CSCs, osteosarcoma CSCs and breast CSCs have recently been shown to be a target for parthenolide, as demonstrated *in vitro* [199-201] and in xenograft mice [46, 202]. In addition to its ability to inhibit NF-κB, parthenolide has been shown in prostate CSCs to target the non-receptor tyrosine kinase src and its downstream signaling components as well as a variety of transcription factors critical for initiation, progression and metastasis of prostate cancer [202]. As a consequence of the promising activity of parthenolide and its analogs against AML SCs and primary AML cells [197, 198], a novel dimethylamino-parthenolide termed LC-1 was recently developed and is currently in a phase I clinical trial for the treatment of patients with AML [203, 204].

4.2.2. Cyclopamine

The steroidal alkaloid cyclopamine (11-deoxojervine) isolated from California corn lily (*Veratrum californicum*) has both teratogenic and antitumor activities arising from its ability to inhibit cellular responses to vertebrate Hedgehog signaling

[205, 206]. Cyclopamine directly binds to the heptameric bundle of smoothened (Smo), a transmembrane serpentine receptor of the proximal Hedgehog signaling pathway [126, 207]. The Hedgehog pathway is a major regulator of many fundamental processes in vertebrate embryonic development, including stem cell maintenance, cell differentiation, tissue polarity and cell proliferation [208], and constitutive or inappropriate activation of the Hedgehog pathway is observed in a wide variety of human cancers [209].

Recent findings suggest that the Hedgehog pathway regulates the maintenance and proliferation of CSCs and promotes carcinogenesis and tumor invasiveness [20, 210]. A number of recent studies have shown that inhibition of the proximal Hedgehog signaling pathway by cyclopamine can eliminate CSCs *in vitro* and in xenograft mice. In particular, expression of the Hedgehog target transcription factor Gli1 is markedly decreased in human glioblastoma CSCs in response to treatment with cyclopamine [211], and formation of tumorspheres initiated by glioblastoma CSCs is completely inhibited by cyclopamine [211, 212], whereas radiation of the tumorspheres results in an enrichment of glioblastoma CSCs [211]. Importantly, human glioblastoma CSCs pretreated with cyclopamine are no longer able to establish intracerebral glioblastoma tumors in mice [211, 212], demonstrating that the Hedgehog signaling pathway is essential for CSC-driven glioblastoma tumorigenesis, which can be effectively blocked by the Hedgehog inhibitor cylopamine. The Hedgehog signaling pathway is also essential for the maintenance and self-renewal of CSCs in multiple myeloma (MM) and chronic myeloid leukemia (CML), as demonstrated by the ability of cyclopamine to inhibit the growth of MM and CML SCs *in vitro* [22, 213, 214] and to eradicate human CML SCs and drug-resistant CML cells in xenograft mice [22, 214]. In addition, cyclopamine has recently been shown to eliminate *in vitro* gastric, hepatoma and prostate CSCs [44, 215, 216] and to inhibit in mice the tumorigenicity of human breast CSCs [217]. Because cyclopamine has low affinity for Smo and displays poor oral bioavailability, suboptimal pharmacokinetics and low metabolic stability, IPI-926, a semisynthetic cyclopamine analog with improved potency, oral bioavailability and a favorable pharmacokinetic profile relative to cyclopamine was designed and synthesized [218]. IPI-926 (saridegib) has been demonstrated to inhibit self-renewal in B-cell acute lymphocytic leukemia SCs [219] and to increase vascularization-mediated gemcitabine delivery and disease stabilization in xenograft mice with gemcitabine-resistant human pancreatic cancer [220]. IPI-926 is currently in a phase I clinical trial in patients with advanced solid tumors (http://www.cancer.gov/clinicaltrials).

4.2.3. Epigallocatechin-3-Gallate (EGCG)

Epigallocatechin-3-gallate (EGCG) is the major and most abundant catechin in green tea, a commonly consumed beverage derived from the dried leaves of the plant *Camellia sinensis* [221, 222]. Chemically, EGCG belongs to the large family of plant-derived polyphenols and constitutes a powerful radical scavenger that exhibits strong antioxidant activities due to the presence of phenolic groups that are sensitive to oxidation and that can generate quinones [222, 223]. EGCG and related green tea polyphenols have been demonstrated to inhibit carcinogenesis by affecting a wide array of signal transduction pathways in premalignant and malignant cells, to induce apoptosis and cell cycle arrest selectively in cancer cells and to inhibit epigenetic modifications, angiogenesis, carcinogenesis and metastasis in mice [221, 222, 224]. Moreover, EGCG can alter microRNA (miR) expression profiles leading to inhibition of cancer cell growth, reversal of EMT and enhancement of the efficacy of conventional anticancer drugs [225, 226]. A large number of studies in animals as well as epidemiologic and case-control studies in humans reveal that long-term green tea consumption or oral administration of EGCG significantly reduces the incidence of various cancers [221, 227], pointing out a pivotal role for EGCG in the chemoprevention of cancer.

In CSCs isolated from human prostate cancer cell lines and primary prostate tumors, EGCG inhibits the self-renewal capacity and induces caspase-dependent apoptosis of CD44+ CD133+ prostate CSCs, as determined in tumorsphere assays [228]. Furthermore, EGCG has been shown to inhibit in prostate CSCs the expression of anti-apoptotic proteins, such as Bcl-2, survivin and XIAP, and, most notably, EGCG was able to inhibit EMT of the cells, as demonstrated by the inhibition of expression of the EMT-associated proteins vimentin, nuclear β-catenin, Slug and Snail [228]. In a follow-up study, similar results were obtained in pancreatic CSCs. It was shown that EGCG inhibits in pancreatic CSCs the pluripotency-maintaining transcription factors Nanog, c-Myc and Oct4 as well as key signaling components of the Hedgehog pathway, including Smo, Patched, Gli1 and Gli2 [229], finally

suggesting that EGCG is capable of inhibiting multiple specific components and pathways in CSCs. EGCG and green tea extracts are currently in phase I-II clinical trials in patients with metastatic prostate cancer, small cell lung cancer and advanced solid tumors (http://www.cancer.gov/clinicaltrials). Very recently, synthetic analogs of EGCG have been developed that display a more potent activation of adenosine monophosphate-activated protein kinase (AMPK) than EGCG or metformin (see below). Activation of AMPK by these EGCG analogs resulted in inhibition of cell proliferation, up-regulation of the cyclin-dependent kinase inhibitor p21, down-regulation of the mTOR pathway, and elimination of stem cell populations in human breast cancer cells [230].

4.2.4. Resveratrol

Resveratrol (3,5,4´-trihydroxystilbene) is a stilbenoid, a type of a natural polyphenol that is found in highest concentration in the skins of red wine grapes (*Vitis vinifera*), in red wine and in sprouted peanuts (*Arachis hypogaea*). The compound shows diverse biochemical activities, including anti-inflammatory, anti-oxidative, anti-proliferative and cancer chemopreventive effects [231, 232]. Experimental studies have demonstrated that resveratrol inhibits the growth of various cancer cells by induction of cell cycle arrest, apoptosis, autophagy and mitotic catastrophe [231, 233], and, like other phytochemicals and natural agents such as EGCG and curcumin, resveratrol interferes with the Wnt and Hedgehog signaling pathways and modulates the expression profiles of miRs [225, 234].

It was recently shown that resveratrol inhibits proliferation and exhibits selective cytotoxicity in CSCs enriched from human medulloblastoma cells and breast cancer cells [235, 236]. In CSCs derived from human glioblastoma cells, resveratrol is able to promote glial-like and neuronal-like differentiation [237] and to induce cell cycle arrest, autophagy, apoptosis and inhibition of tumorsphere formation of the cells [238]. Moreover, in pancreatic CSCs derived from human primary tumors, resveratrol induces apoptosis, inhibits EMT, reverses multi-drug resistance and suppresses the self-renewal capacity of the cells that is accompanied by the modulation of expression of key regulatory proteins, such as Bcl-2, XIAP, Zeb-1, Slug, Snail, ABCG2, Nanog, Sox-2, c-Myc and Oct4 [239]. These multiple effects of resveratrol against pancreatic CSCs finally lead to the inhibition of the development and growth of pancreatic cancer in $Kras^{G12D}$ mice prone to spontaneously develop invasive and metastatic pancreatic ductal adenocarcinoma [239]. Resveratrol has recently been shown to modulate the expression profiles of miRs that regulate the expression of tumor suppressors or oncogenic proteins. In particular, levels of tumor suppressive miRs, such as miR-622 and miR-633 are up-regulated, while several oncogenic miRs targeting effectors of the TGFβ signaling pathway are down-regulated by resveratrol in cancer cells [240-242], providing a mechanistic basis for the anti-cancer activity of resveratrol. To date several clinical studies are underway to evaluate the efficacy of resveratrol in cancer prevention [243]. Data from one of such study have shown that resveratrol can prevent the development of colon cancer by inhibiting Wnt pathway target gene expression in colonic mucosa [244].

4.2.5. Curcumin

The rhizomes of the Indian spice plant turmeric (*Curcuma longa*) contain high concentrations of the natural polyphenol curcumin, which is known to exhibit anti-cancer and chemopreventive activities in humans [245-247]. As revealed by molecular interaction studies, curcumin displays pleiotropic biochemical activities, such as anti-carcinogenic, anti-angiogenic, anti-microbial, anti-viral, anti-inflammatory, anti-oxidant, pro-apoptotic, chemosensitization, radiosensitization and immunomodulatory activities [248-249]. The underlying mechanisms of these activities involve the binding of curcumin to a variety of biomolecules and the regulation of multiple molecular targets, including transcription factors, signaling proteins, growth factors, adhesion molecules, cell cycle regulatory proteins, carrier proteins, protein kinases, inflammatory cytokines, chemokines and their receptors, matrix metalloproteinases, metal ions, miRs and proteasomes that may provide a pharmacological basis for a multi-targeted cancer therapy [225, 234, 248-250].

Initial studies using rat C6 glioma cells revealed that curcumin is able to inhibit the viability and growth of glioma CSCs sorted from C6 cells by virtue of the ability to exclude the DNA binding dye Hoechst 33346, a hallmark of cancer stem-like cells belonging to the so called side population [251]. A more extended study subsequently showed that curcumin promotes, *in vitro* and in xenograft mice, differentiation of glioblastoma CSCs by inducing autophagy [252]. In response to treatment with curcumin, glioblastoma CSCs obtained from surgically resected human glioblastomas expressed high levels of differentiation markers (Tuj1, GFAP, Olg2 and βIII-tubulin) and low levels of neural

stem/progenitor markers (CD133 and Nestin) [252]. Curcumin treatment also decreased the amount and size of newly formed tumorspheres and the total number of cells in a clonogenic survival assay, indicating that curcumin represses self-renewal of glioblastoma CSCs. *In vitro* and in intracranial xenograft tumors in mice, curcumin was able to induce autophagy of glioblastoma cells that occurred as a terminal event of differentiation of the cells. Finally, mice bearing intracranial glioblastoma xenografts induced by human glioblastoma CSCs showed intracerebral tumor regression and increased overall survival in response to curcumin treatment, suggesting significant therapeutic potential of curcumin in glioblastoma [252]. Moreover, curcumin inhibits self-renewal of breast CSCs isolated from human MCF-7 breast cancer cells, as demonstrated by the inhibition of tumorsphere formation of breast CSCs treated with curcumin that was accompagnied by inhibition of the Wnt signaling pathway in the cells [253]. Curcumin has also been shown to eliminate colon CSCs generated by continuous exposure of human colon cancer cells to 5-fluoruracil and oxaliplatin (FOLFOX), a chemotherapeutic regime commonly used for the treatment of colon cancer [254]. However, the combination of curcumin and dasatinib, a tyrosine kinase inhibitor used for the therapy of CML and advanced colon carcinoma, was more effective in eliminating FOLFOX-resistant colon CSCs than the single agent [150].

Because of the low bioavailability of curcumin [248], orally available curcumin analogs with improved bioavailability, such as GO-Y030, GO-YY078 and diflourinated curcumin (CDF) have been synthesized recently [255-257]. The curcumin analog GO-Y030 has been shown to inhibit colorectal carcinogenesis in mice harboring a germ-line mutation of adenomatous polyposis coli (APC) [255]. GO-Y030 also eliminates human colon CSCs and tumor growth in xenograft mice [258]. Similar results were obtained using the curcumin analog CDF in human pancreatic CSCs and in human pancreatic cancer xenograft mice [256, 259].

In view of these promising results, a number of clinical studies showing a significant anti-cancer effect of curcumin alone or in combination with conventional cytostatic drugs have been conducted in patients with advanced pancreatic and breast cancer [110, 111, 113, 260]. Of note, a recently conducted phase IIa clinical trial reveals that curcumin can prevent the development of colorectal cancer [247].

4.2.6. Sulforaphane

The anticarcinogenic activities of sulforaphane, a natural isothiocyanate found in broccoli (*Brassica oleracea italica*), have been detected on the basis of its ability to induce phase II detoxication enzymes, such as quinone reductase and glutathione *S*-transferase in mouse hepatoma cells, and to prevent the formation of mammary tumors in rats [261]. Subsequently, it was demonstrated that sulforaphane specifically binds to transcriptionally active c-Rel-containing NF-κB complexes and inhibits NF-κB-mediated antiapoptotic signaling in human CD24+ CD44- pancreatic CSCs, thereby inducing apoptosis and preventing clonogenic growth and tumorsphere formation of the cells [262]. In human pancreatic cancer xenograft mice, sulphoraphane treatment markedly blocked tumor growth and angiogenesis that was further enhanced by additional treatment with tumor necrosis factor related apoptosis inducing ligand (TRAIL) [262]. Similar results were obtained in a study using breast CSCs sorted from human breast cancer cell lines [263]. In this study, sulforaphane was able to inhibit breast CSC tumorsphere formation, to deplete human breast CSCs in xenograft mice, and to abrogate tumor growth after the reimplantation of tumor cells from sulforaphane-treated mice into secondary mice. As also shown for salinomycin and oxymatrine (see *4.2.7*) in other studies [139, 264, 265], sulforaphane down-regulated in human breast CSCs the Wnt/β-catenin signaling pathway [263], which is essential for survival, self-renewal and therapy resistance of human CSCs [20, 21]. Interestingly, sulforaphane acts synergistically with the multikinase inhibitors sorafenib and imatinib, the plant-derived flavonoid quercetin or with convetional cytostatic drugs, such as cisplatin, gemcitabine, doxorubicin and 5-fluorouracil to eliminate pancreatic, prostate and leukemia CSCs *in vitro* and to inhibit initiation and growth of human pancreatic cancer in xenograft mice [151, 152, 266, 267]. Similar synergistic effects have been observed for the combination of salinomycin and imatinib [143], salinomycin and doxorubicin [141], and curcumin and dasatinib [150], suggesting that agents targeting CSCs have the potential to act synergistically with conventional chemotherapeutic drugs and novel tumor-targeted drugs.

4.2.7. Oxymatrine

Oxymatrine is a quinolizidine alkaloid found in the dried roots of the small tree *Sophora flavescens*. Besides oxymatrine, the roots of *Sophora flavescens* (Chinese: *Ku shen*) contain other bioactive

quinolizidine alkaloids, including matrine, sophoridine, sophocarpine, aloperine and cytosine [268]. Extracts of *Ku shen* have been used for centuries in traditional Chinese medicine as herbal formulations for the treatment of cancer, liver and skin disorders, cardiac arrhythmia, leukopenia and bronchitis [269]. Oxymatrine has recently been shown to induce apoptosis, cell cycle arrest, and the generation of reactive oxygen species in a variety of human cancer cells, including colon cancer, melanoma and pancreatic cancer cells [270-272]. Subsequent studies revealed that oxymatrine eliminates human breast CSCs by down-regulating the Wnt/β-catenin signaling pathway [264, 265]. In particular, oxymatrine was able to eliminate breast CSCs sorted from human MCF-7 breast cancer cells and to inhibit the expression of the main genes of the Wnt/ β-catenin pathway (*β-catenin*, *cyclin D1* and *c-myc*) in the cells [264, 265]. In contrast to the chemotherapeutic drug cisplatin, oxymatrine induced significant regression of human MCF-7 breast cancers in xenograft mice that was accompanied by the down-regulation of expression of key signaling proteins of theWnt/β-catenin pathway (Wnt1, β-catenin, c-Myc, Cyclin D1, LEF1 and TCF4), indicating that oxymatrine eradicates breast CSCs by targeting the Wnt/β-catenin signaling pathway [265].

4.3. Small-Molecule Inhibitors

4.3.1. Vismodegib (GDC-0449)

Vismodegib (GDC-0449) is a synthetic low-moelcular weight Hedgehog pathway inhibitor that acts as a receptor antagonist of smoothened (Smo), a serpentine receptor of the proximal Hedgehog signaling pathway [125, 209, 273].

The orally bioavailable compound, which has been developed and optimized by high-throughput screening [273], is structurally unrelated to other Smo inhibitors, such as cyclopamine and IPI-926 (see *4.2.2.*) and has been demonstrated to be ~10 times more potent than cyclopamine at inhibiting Hedgehog pathway activity [274]. Vismodegib effectively eliminates mouse medulloblastoma and hepatocellular carcinoma [273-275] as well as human pancreatic and colon cancers in xenograft mice [276].

These findings rapidly led to the initiation of clinical trials with vismodegib in patients with cancers exhibiting constitutive up-regulation of the Hedgehog signaling pathway as a result of mutations in the *Patched 1* or *Smo* gene [125, 209]. Such mutations are found in patients with Gorlin´s syndrome that have a marked susceptibility to develop basal cell carcinomas (BCC) and medulloblastomas [125, 209]. Accordingly, two phase I studies revealed that vismodegib has encouraging antitumor activity in patients with BCC and advanced solid tumors refractory to current therapies [277, 278]. Vismodegib is currently undergoing phase II clinical trials for the treatment of advanced BCC, medulloblastoma, glioblastoma multiforme, chondrosarcoma, and ovarian, pancreatic, gastric, lung, breast and colorectal cancer [125, 279], (http://www.cancer.gov/clinicaltrials).

As in the case of the Smo/Hedgehog signaling pathway inhibitor cyclopamine and its analog IPI-926 (see *4.2.2.*), vismodegib is able to target CSCs [280, 281]. In pancreatic CSCs derived from human primary tumors, vismodegib has recently been shown to inhibit cell viability and to induce apoptosis that is accompanied by caspase-3 activation, PARP cleavage, expression of the death receptors Fas, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), and by down-regulation of Bcl-2 expression [280]. Moreover, vismodegib inhibited the expression of the Hedgehog pathway receptors Patched 1, Patched 2 and Smo in pancreatic CSCs. It was also demonstrated that human pancreatic CSCs require the activity of the Hedgehog pathway transcription factors Gli1 and Gli2 for sustained expression of genes critical for cell survival and proliferation. However, vismodegib inhibited nuclear translocation, DNA binding and transcriptional activity of Gli1 and Gli2 in pancreatic CSCs, indicating that vismodegib targets pancreatic CSCs by inhibiting various components of the Hedgehog signaling pathway [280]. Vismodegib has also been shown to inhibit cell growth of human lung adenocarcinoma cells and small cell lung carcinoma cells, but Smo, the molecular target of vismodegib, is not expressed in these cells [281]. By contrast, cancer stem-like cells (side population) sorted from the lung cancer cells markedly express Smo and are effectively eliminated by vismodegib [281], suggesting that the anticancer activity of vismodegib in lung cancer is mediated by selective targeting lung CSCs.

4.3.2. Repertaxin

Repertaxin is a small-molecule noncompetitive allosteric inhibitor of CXCR1 (interleukin-8 receptor-α) and CXCR2 (interleukin-8 receptor-β), which both play a major role in mediating interleukin-8-dependent chemotaxis and migration of polymorphonuclear leukocytes, T lymphocytes and natural killer cells [282, 283]. By inhibiting interleukin-8-mediated activities,

such as recruitment and activation of leukocytes in inflammatory sites, repertaxin is able to attenuate ischemic organ damages and reperfusion injury in rodents [282, 284].

A recent study revealed that CXCR1 blockade by repertaxin selectively targets human breast CSCs *in vitro* and in xenograft mice [124]. Repertaxin was able to selectively deplete the CSC population in human breast cancer cells and to markedly decrease the ability of breast CSCs to form tumorspheres. The significant decrease of breast CSC viability induced by repertaxin was mediated by Fas/Fas ligand-induced apoptosis and by interference with the FAK/Akt/FOXO3A survival pathway, which is critical for the maintainenance and viability of CSCs [124]. In contrast to the chemotherapeutic drug docetaxel, repertaxin was able to eliminate the CSC population in human breast cancer xenograft mice, leading to the reduction of both tumor growth and systemic metastasis [124]. In view of more recent data showing that IL-8 can promote EMT, tumor growth, angiogenesis, metastasis and chemotherapeutic drug resistance [285, 286], targeting IL-8- and CXCR1-mediated regulatory pathways by repertaxin may represent a promising strategy to eliminate breast CSCs.

4.4. Classical Drugs

4.4.1. Metformin

Metformin (dimethylbiguanide) is a biguanide developed from galegine, a guanidine derivate found in the plant French lilac (*Galega officinalis*) [287]. Metformin constitutes an oral anti-diabetic drug of the biguanide class that is used for decades as the most effective drug in the first-line treatment of type 2 diabetes mellitus [288]. The drug displays a variety of biological and biochemical activities, including improvement of hyperglycemia by suppressing hepatic gluconeogenesis, reduction of circulating insulin levels, activation of adenosine monophosphate-activated protein kinase (AMPK), inhibition of insulin-like growth factor (IGF) and PI3K/Akt signaling activities, suppression of HER2 (erbB-2) oncoprotein overexpression, inhibition of several protein kinases and receptor tyrosine kinases, inhibition of the mammalian target of rapamycin (mTOR) pathway and its downstream effectors, the ability to reverse EMT, and activation of an atypical protein kinase C-CBC pathway resulting in mammalian embryonic and adult neurogenesis [133, 289-294].

In human cancer cells of different origin, metformin has been shown to induce cell cycle arrest, growth inhibition and apoptosis through interfering with various signaling pathways [295-298]. Moreover, metformin inhibits tumor growth of human colon, prostate, breast and lung cancer in xenograft mice [295, 297, 299, 300]. Epidemiological studies reveal that metformin significantly reduces cancer incidence and improves cancer survival in patients with type 2 diabetes [301, 302], indicating that metformin is a potent anti-cancer and chemopreventive drug.

As demonstrated in a number of recent studies, metformin is able to selectively target CSCs in different types of human cancers, including breast [90, 130, 131, 303, 304], pancreatic [305] and thyroid cancer [306]. In particular, metformin has been shown to selectively kill CSCs in four genetically different types of human breast cancer, to inhibit tumorsphere formation of the breast CSCs and to reduce tumor growth and relapse in human breast cancer xenograft mice [130]. In human basal-like breast cancer cells naturally enriched for $CD44^{high}/CD24^{low}$ CSC populations, metformin significantly reduced the proportion of $CD44^{high}/CD24^{low}$ cells and transcriptionally repressed the expression of EMT-related gene products, such as the transcription factors ZEB1, TWIST1 and Slug and the pleiotropic cytokines TGFβ1-3, thereby repressing the activation of the genetic EMT program in breast CSCs [303]. In trastuzumab-resistant human breast CSCs, metformin suppresses self-renewal and proliferation, as demonstrated by suppression of tumorsphere formation in response to metformin treatment. Interestingly, metformin was able to overcome trastuzumab resistance and to synergistically interact with trastuzumab to more effectively suppress self-renewal and proliferation of the cells *in vitro* [131]. A subsequent study shows that metformin induces marked tumor regression in xenograft mice with human primary trastuzumab-resistant HER2+ breast cancer. In this xenograft model, metformin was able to overcome primary resistance to trastuzumab in HER2+ human breast cancer by selectively killing CD44+/CD24-/low breast CSCs [90]. Metformin has also been shown to overcome the resistance to radiation of human MCF-7 breast cancer cell-derived CSCs [307]. In MCF-7-derived $CD44^{high}/CD24^{low}$ CSCs, which display increased tumorsphere formation and expression of the stem cell marker Oct4 in response to treatment with17-β-estradiol, metformin was able to inhibit self-renewal and proliferation of the cells [304]. Moreover, metformin inhibited 17-β-estradiol-induced expression of Oct4 in

the $CD44^{high}/CD24^{low}$ breast CSCs, finally leading to the inhibition of tumorsphere formation [304]. A recent study elegantly demonstrates that metformin targets human pancreatic CSCs by interfering with the expression of key transcription factors of essential self-renewal and maintenance programs of embryonic stem cells and CSCs, including Notch-1, Nanog, Oct4 and EZH2 [305]. It was demonstrated that metformin decreased the mRNA levels of these transcription factors in pancreatic CSCs and simultaneously caused the re-expression of miRs of the let-7 and miR-200 family, which are typically lost in pancreatic CSCs, leading to the maintenance of the CSC state. These abilities of metformin to directly modulate the levels of key regulators of CSCs function may result in the inhibition of self-renewal, proliferation, migration and invasion of pancreatic CSCs, as demonstrated in the study [305]. Finally, metformin has recently been shown to suppress self-renewal of human thyroid cancer stem-like cells and to kill thyroid CSCs derived from doxorubicin-resistant and Oct4-expressing thyroid cancer cells [306], indicating that metformin is able to target CSCs in different types of human cancers. All these promising result have ultimately led to the initiation of phase I-III clinical trials with metformin alone or in combination with conventional chemotherapeutic drugs in patients with breast cancer [112, 308] and various other solid tumors [115, 133], (http://www.cancer.gov/clinicaltrials).

4.4.2. Tranilast

Tranilast [*N*-(3´,4´-dimethoxycinnamoyl)-anthranilic acid] is a synthetic cinnamoyl anthranilate that has been in clinical use in Japan since 1982 for the treatment of allergic and fibrotic diseases [309-311]. Its safety, tolerability and efficacy in the treatment of allergic rhinits, bronchial asthma, atopic dermatitis, coronary restenosis and various fibrotic diseases has been studied in several thousand patients receiving up to 600 mg/day for months [309, 310]. The drug exhibits various mechanisms of action, including inhibition of mast cell and basophil degranulation, inhibition of collagen synthesis, inhibition of TGF-β production and receptor expression, inhibition of Smad2 and ERK phosphorylation, inhibition of synthesis of IL-6, IL-12, IFN-γ and PGE2, and antagonization of the effects of VEGF [310, 311].

It was early shown that tranilast inhibits the proliferation of human malignant glioma cells *in vitro* and of rat gliomas *in vivo* by reducing the expression and release of TGF-β [312]. In addition, recent studies demonstrate marked anti-cancer activities of transilast in breast and prostate cancer cells *in vitro* and in mice [313-315]. Of note, data from a clinical pilot study indicate that tranilast improves prognosis and survival of patients with advanced prostate cancer [316].

Tranilast is also able to inhibit tumorsphere formation of human breast CSCs isolated from highly aggressive mitoxantrone-resistant triple negative (estrogen receptor negative, progesterone receptor negative, and HER2 negative) or triple positive breast cancer cells [134], (Figure **2**). Inhibition of tumorsphere formation by tranilast is acompagnied by decreased retinoblastoma protein phosphorylation and decreased expression of the stem cell markers Oct4 and CD133. Tranilast is also effective *in vivo* since it prevents lung metastasis in xenograft mice injected with human mitoxantrone-resistant triple negative breast cancer cells [134]. Finally, the study revealed that tranilast is an agonist of the aryl hydrocarbon receptor (AHR), which is expressed in breast CSCs and which mediates the anti-proliferative and anti-CSCs activities of tranilast [134]. These data may indicate that tranilast as an established and safe drug can be used as an agent against breast CSCs and aggressive breast cancer in clinical situations.

4.4.3. Thioridazine

The antipsychotic drug thioridazine (10-[2-(1-methyl-2-piperidy) ethyl]-2-methylthiophenothiazine) is a piperidine phenothiazine that acts as a dopamine D2 receptor antagonist and that has been used for decades for the treatment of schizophrenia and other psychosis [317, 318]. It was early shown that thioridazine inhibits proliferation and overcomes cytostatic drug resistance of human breast cancer cells [319, 320], and recent studies reveal that thioridazine induces cell cycle arrest and apoptosis in human cancer cells by targeting the PI3K/Akt/mTOR pathway [321, 322]. Notably, schizophrenic patients treated with dopamine receptor antagonists such as thioridazine were reported to have a reduced incidence of colorectal and prostate cancer compared to the general population [323], suggesting a chemopreventive role for thioridazine and other dopamine antagonists.

As elegantly demonstrated in a recent study, thiorodazine is capable of targeting neoplasic human pluripotent stem cells (hPSCs) derived from human embryonic stem cells as well as targeting human AML SCs [135]. In particular, thioridazine induces differentiation of neoplastic hPSCs by down-regulation

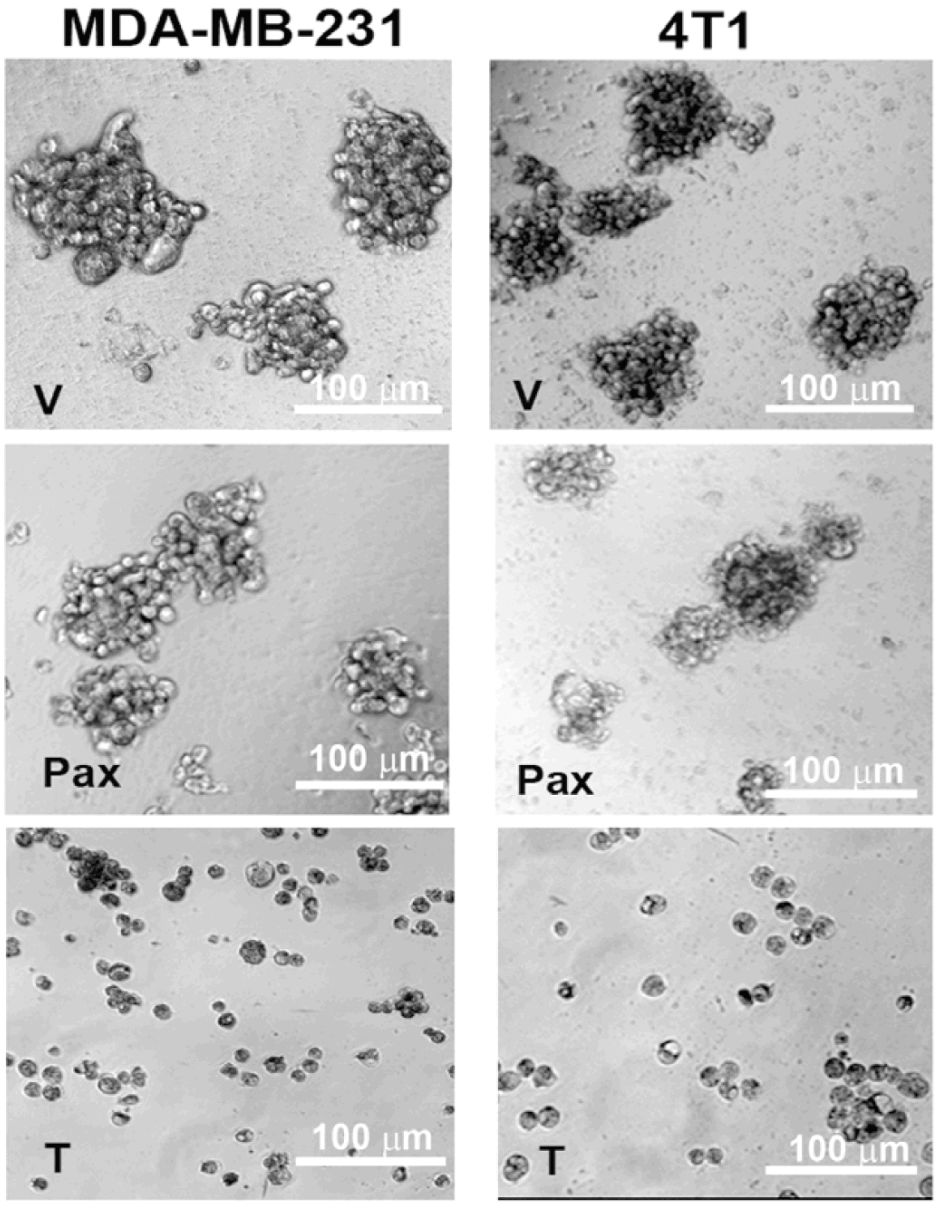

Figure 2: Tranilast (T), but not paclitaxel (Pax) inhibits tumorphere formation of highly aggressive mitoxantrone-resistant triple negative human (MDA-MB-231) and mouse (4T1) breast cancer cells. After tumorsphere formation for 7 days, Tranilast (200 μM) or paclitaxel (20 nM) were added for 48 h to the cultures. V: vehicle. Adapted from [134], with permission from www.plosone.org.

of Oct4 expression and up-regulation of the expression of various differentiation-associated genes. In hematopoietic colony forming assays, thioridazine selectively eliminates human AML blasts expressing dopamine D2 receptors, but does not affect normal human hematopoietic stem and progenitor cells, which lack expression of dopamine D2 receptors [135]. Thioridazine significantly eliminates human AML SCs, but does not affect normal hematopoiesis sustained by human hematopoietic stem and progenitor cells in xenograft mice [135]. Because thioridazine exerts its anti-CSC activity *via* antagonism of D2-family dopamine receptors expressed on neoplastic hPSCs, human AML blasts, AML SCs and triple negative breast cancer cells [135], D2 dopamine receptor signaling may represent a druggable receptor pathway in human CSCs and cancer cells, and established and safe dopamine antagonists such as thioridazine might be used for the elimination of CSCs in future clinical trials.

4.5. Monoclonal Antibodies

4.5.1. H90 (Anti-CD44), and P245 (Anti-CD44)

H90 is a mouse monoclonal IgG1 antibody directed against human CD44 [324], a transmembrane glycoprotein and the receptor for hyaloronic acid,

osteoponitin, collagens, fibronection, selectin and laminin. CD44 mediates adhesive cell to cell and cell to extracellular matrix interactions through binding to hyaloronic acid and its other ligands [325]. CD44 is expressed on leukemic blasts in all human AML subtypes and plays an important role in the regulation of normal and malignant myelopoiesis [326, 327]. Ligation of CD44 by H90 activates CD44 signaling, reverses differentiation blockage and induces myeloid differentiation in AML blasts of subtypes M1 to M5 obtained from patients [328]. H90 also inhibits proliferation, induces terminal differentiation and mediates apoptosis in human myeloid leukemia cell lines [329, 330]. CD44 is abundantly expressed on CSCs in hematopoietic and epithelial malignancies and fulfils some of the special properties that are displayed by CSCs [331]. Therefore, targeting CD44 by activating monoclonal antibodies appears as a reasonable strategy to eliminate CSCs.

In fact, H90 is the first monoclonal antibody that has been shown to target CSCs. As demonstrated in a seminal study in 2006, H90 induces terminal differentiation and inhibits engraftment, homing, proliferation and the repopulation capacity of human AML SCs in a NOD-SCID mouse xenograft model [332]. This study reveals for the first time that CD44 is a key regulator of AML SC function that is essential for proper homing of AML SCs to microenvironmental niches and for maintaining AML SCs in a primitive state [332].

Another mouse monoclonal IgG1 antibody raised against human CD44 is P245, which has been shown to reduce tumor growth and to eliminate breast CSCs in xenograft mice with human triple negative basal-like breast cancer [333]. Triple negative basal-like breast cancer cells resemble many features of breast CSCs, including expression of $CD44^{high}$, $CD24^{low}$, ALDH1, and the triple negative basal-like subtype of breast cancer is characterized by a high content of breast CSCs, aggressive proliferation, high metastatic capability and poor overall survival of patients [334-336]. P245 is able to significantly reduce tumor growth in xenograft mice with human triple negative basal-like breast cancer [333]. Treatment of the mice with doxorubicin and cyclophosphamide, a cytostatic drug combination commonly used for the therapy of triple negative basal-like breast cancer, resulted in complete histological tumor regression, but residual breast CSCs survived the doxorubicin/cyclophosphamide therapy and could be detected by virtue of their CD44 expression [333]. Tumor relapse mediated by the residual CD44 breast CSCs occurred 4-6 weeks after complete histological regression, but the relapse could be effectively prevented when P245 was systemically injected during the tumor regression period [333]. These data provide substantial evidence that anti-CD44 monoclonal antibodies such as P245 can eliminate human breast CSCs and can prevent relapse of aggressive breast cancer.

4.5.2. B6H12.2 (Anti-CD47), and 7G3 (Anti-CD123)

B6H12.2 is a mouse monoclonal IgG1 antibody that binds to and blocks human CD47, a widely expressed transmembrane protein and a receptor for thrombospondin family members that also serves as the ligand for signal regulatory protein alpha (SIRPα) [337-339]. SIRPα is expressed on phagocytic cells including macrophages and dendritic cells, that when bound and activated by CD47 initiates a signal transduction cascade resulting in inhibition of phagocytosis [339-340]. Therefore, the CD47/SIRPα interaction has been attributed as a tool that provides a "don´t eat me"-signal [341].

A seminal study revealed that CD47 is abundantly expressed on human AML SCs and that CD47 is much more highly expressed on AML SCs than on their normal counterparts, such as hematopoietic stem cells (HSCs) and multipotent progenitor cells [342]. As investigated in a large cohort of AML patients, increased CD47 expression in human AML is associated with poor clinical outcome and worse overall survival, providing evidence that increased CD47 expresion on AML SCs substantially contributes to the pathogenesis and fate of human AML [342]. It was furher shown in this study that the CD47-blocking monoclonal antibody B6H12.2 preferently enables phagocytosis of human AML SCs by human and mouse macrophages [342]. Similar results were obtained in a study with human CD47-expressing bladder CSCs [343]. In a xenograft mouse model, B6H12.2 prevented the engraftment of human AML SCs, and treatment of human AML-xenograft mice with B6H12.2 completely eradicated AML cells by the mechanism of phagocytosis *in vivo*, whereas normal HSCs were not depleted [342]. Thus, human AML SCs can be targeted and eradicated with blocking anti-CD47 antibodies such as B6H12.2 capable of enabling phagocytosis of AML SCs.

The mouse monoclonal IgG2a antibody 7G3 recognizes the *N*-terminal domain of the human interleukin-3 (IL-3) receptor α chain (CD123) and

functions as a specific IL-3 receptor antagonist that can antgonize IL-3 biologic activities, such as histamine release from basophil granulocytes or IL-6 and IL-8 secretion from endothelial cells [344]. In comparison to normal HSCs, CD123 is overexpressed on AML blasts, CD34+ leukemic progenitors and AML SCs, and CD123 confers growth advantage of AML cells over HSCs [345-347]. Clinically, high CD123 expression in AML is associated with higher blast counts at diagnosis and a lower complete remission rate that results in poor prognosis and reduced survival [346, 348], ultimately defining CD123 as a promising cell-surface target for the elimination of AML SCs and the eradication of AML.

In fact, the CD123-targeting antibody 7G3 has recently been shown to eliminate human AML SCs [347]. *Ex vivo* treatment of primary human AML cells with 7G3 selectively inhibited engraftment, repopulation ability and bone marrow and spleen homing of the cells in NOD/SCID mice, whereas 7G3 treatment of normal HSCs derived form human cord blood or bone marrow resulted in significant engraftment and hematopoietic differentiation of the human HSCs in NOD/SCID mice. Moreover, 7G3 selectively eradicated human AML SCs in NOD/SCID mice engrafted with primary human AML cells. It was further demonstrated that 7G3-mediated inhibition of engraftment and homing of AML SCs in NOD/SCID mice is dependent on antibody dependent cellular cytotoxicity (ADCC) induced by the Fc fragment of 7G3 and that 7G3 inhibits spontaneous and IL-3-induced proliferation of AML SCs *in vitro*. These pleiotropic activities of 7G3 against human AML cells and AML SCs finally led to the reduction of AML burden and to an improved long-term survival of NOD/SCID mice engrafted with human AML [347]. Consequently, CSL362, a humanized monoclonal antibody that targets CD123 is currently in a phase I clinical trial in patients with AML (http://www.cancer.gov/clinicaltrials).

4.5.3. MT110 (Anti-EpCAM/-CD3, Bispecific, Bifunctional), and Catumaxomab (Anti-EpCAM/-CD3, Bispecific, Trifunctional)

MT110 is bispecific bifunctional single-chain antibody construct of the BiTE (bispecific T cell engager) class that binds to epithelial cell adhesion molecule (EpCAM, CD326) and to the T cell receptor protein complex CD3 [349]. MT110 activates and redirects resting human peripheral CD4+ and CD8+ T cells to induce specific lysis and apoptosis of target cells expressing EpCAM, a transmembrane glycoprotein expressed by CSCs and epithelial cancer cells [349-351].

Initially, it was demonstrated that MT110 is able to eradicate human colon cancer cells and patient-derived metastatic ovarian cancer in NOD/SCID xenograft mice [349]. Moreover, *ex vivo* treatment with MT110 of malignant pleural effusions obtained from patients with advanced breast cancer resulted in a specific lysis of pleural EpCAM+ breast cancer cells by activated and redirected autologous CD4+ and CD8+ T cells, indicating that breast cancer patients with malignant pleural effusions might benefit from targeted therapy with MT110 [350]. Finally, CSCs isolated from patient-derived primary colon or pancreatic cancers injected together with allogeneic or autologous (donor-derived) peripheral mononuclear cells into NOD/SCID mice were effectively eradicated by MT110, and the CSCs did not establish significant tumor growth in the NOD/SCID xenograft mice treated with MT110 [352, 353]. These promising results suggest that EpCAM-expressing CSCs and cancer cells can effectively be eradicated by MT110. Thus, MT110 is currently in a phase I clinical trial in patients with advanced solid tumors (http://www.cancer.gov/clinicaltrials).

Catumaxomab is a chimeric antibody construct consisting of two half antibodies, each with one light and one heavy chain that originate from parental mouse IgG2a and rat IgG2b isotypes [354]. This antibody construct belongs to a novel family of trifunctional, bispecific antibodies termed Triomabs, and has two binding specifities, one directed against EpCAM and one against the T cell receptor protein complex CD3. With its Fc fragment, catumaxomab additionally binds dendritic cells, macrophages and natural killer cells. Therefore, the anti-tumor activity of catumaxomab results from T-cell-mediated lysis, ADCC, and phagocytosis *via* activation of Fcγ receptor-positive accessory cells. Importantly, no additional activation of immune cells is necessary for effective tumor elimination by catumaxomab, which therefore represents a self-supporting system [355, 356].

Catumaxomab has recently been shown to induce regression of malignant pleural effusions, malignant ascites and peritoneal carcinomatosis in patients with advanced epithelial cancers resistant to conventional chemotherapy [357-359]. In addition, catumaxomab is able to effectively eliminate CD133+/EpCAM+ CSCs from malignant ascites of patients with advanced ovarian, gastric and pancreatic cancer [360], indicating that catumaxomab can be therapeutically used to eradicate CSCs of epithelial cancers. Therefore, catumaxomab is currently in phase I-III clinical trials in patients with advanced ovarian, gastric and non-small

cell lung cancer, and is in the European Union on the market for the therapy of malignant ascites caused by epithelial cancers [361], (http://www.cancer.gov/clinicaltrials).

5. CONCLUSIONS AND FUTURE DIRECTIONS

Work from the last few years highlights the possibility of selectively targeting CSCs, which are regarded as the major culprits in cancer. However, although the rather novel CSC concept of carcinogenesis is fairly accepted to date, more classical mechanisms and driving forces of carcinogenesis, including genome instability, epigenetic modifications, first oncogenic hit(s), clonal evolution, replicative immortality, invasion and metastasis, immune evasion, reprogramming of energy metabolism, and most probably, a complex interplay of all of these mechanisms must be considered as a basis for defining carcinogenesis and cancer in general [99, 106, 362-367]. Nevertheless, in line with the CSC concept of carcinogenesis [1, 3, 4, 7-9, 99], CSCs constitute adequately characterized cells and represent novel and translationally relevant targets for cancer therapy [5, 67, 100, 101].

Significant advances have been made recently in the discovery, development and validation of novel compounds and drugs that target CSCs, and the future clinical use of these novel agents may represent a powerful strategy for eradicating CSCs in cancer patients, thereby preventing tumor recurrence and metastasis, and, hopefully, contributing to the cure of cancer. There is growing consensus that conventional cytotoxic drugs are unable to eradicate CSCs [5, 12, 66, 100], and, more disturbing, CSCs can be even selectively enriched by these drugs, as demonstrated in breast cancer patients receiving systemic chemotherapy comprising conventional cytotoxic drugs [10, 70, 71, 368]. Moreover, many novel tumor-targeted drugs, including tyrosine kinase inhibitors and monoclonal antibodies raised against tumor-specific cell surface proteins, also fail to eliminate CSCs [73, 80, 88, 90, 98], so that there is an urgent need for novel compounds and drugs that effectively target CSCs for the use in elaborated clinical settings, preferably in combination with conventional cytostatic drugs and novel tumor-targeted agents.

In this context, one promising candidate drug is the ionophore antibiotic salinomycin, which has recently been documented to effectively eliminate CSCs in different types of human cancers *in vitro* and in xenograft mice bearing human cancers [116, 117, 142, 170]. It is important to note that salinomycin, in combination with conventional cytotoxic drugs, is much more effective in eradicating human cancers in xenograft mice than the single agent [116, 117]. Similar findings have been obtained in xenograft mice treated with a combination of sulforaphane and sorafenib [151] or curcumin and dasatinib [150] or metformin and paclitaxel [114]. More intriguingly, in phase I and II clinical trials, curcumin in combination with gemcitabine [110, 113] and docetaxel [111] has been proven successful in the treatment of advanced pancreatic and breast cancer, respectively. A recent phase I study of metformin and the mTOR-inhibitor temsirolimus in patients with advanced solid tumors and exhausted standard treatment options revealed similar promising results [115].

This is in accord with the postulation that efficient cancer therapy should target all cancer cell populations, including CSCs, more differentiated progenitors, and bulk tumor cells that might be achieved by combining CSC targeting agents with conventional cytotoxic drugs, novel tumor-targeted drugs and radiation therapy [5, 100, 108]. Finally, the establishment of appropriate biomarkers and the definition of novel clinical endpoints for monitoring the efficacy of such combined therapeutic approaches will be a challenge [369, 370]. More work is required to study in detail the molecular mechanisms, the clinical efficacy, and the long-term safety of compounds and drugs that target CSCs, with the aim of providing novel and highly effective therapies for patients in all stages of cancer.

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