Mutual regulation and targeting of multidrug resistance and cancer stem phenotype

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Abstract

Cancer-initiating cells referred as cancer stem cells (CSCs) retain the essential property of self-renewal and protection. The protective mechanisms enable tumour regrowth even after the application of chemotherapy that was believed to be successful. Among protective mechanisms of CSCs, the overexpression of ATP Binding Cassette (ABC) membrane transporters is highly important. ABC transporters involved in the development of cancer multidrug resistance (MDR) such as P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) are considered as particular features of CSCs. They provide the shield for CSCs and protect them from the adverse effects of chemotherapeutics. Hence, combating MDR would be one of the strategies for the elimination of CSCs. In order to investigate this phenomenon many model systems comprising MDR cancer cells have been established. Some of them were developed by selection process through exposure to various anticancer drugs, others by transfection of genes for ABC transporters, while some were obtained by sorting the side-population considered to possess stemness and resistant phenotype. Herein we review the potential of cancer MDR models for studying CSCs because gaining a better insight into the mechanisms of CSCs resistance to chemotherapy may discover new therapeutic targets and develop better anticancer strategies.

Introduction

Cancer stem cell (CSC) biology is a rapidly developing field within cancer research. Besides stochastic model of carcinogenesis suggesting that each anaplastic cell has tumourigenic potential, widely accepted is CSC model implying that only a small subset of cells contributes to the development of a new tumour (Fig. 1).[1] Thus, cancer stem cells (CSCs) represent a rare population of cells responsible for tumour growth, resistance, and recurrence.[2] The concept of CSCs was first raised by Park et al., in 1971.[3] It was postulated that cancer is a disease driven by a subpopulation of self-renewing CSCs, which possess the ability to generate diverse differentiated cell populations contributing to heterogeneity of tumour.[4] Since then, CSCs have been identified and isolated from tumours of the hematopoietic system, breast, lung, prostate, colon, brain, head and neck, and pancreas.[5]

CSCs phenotype is strongly connected with cancer resistance to radio- and chemotherapy[6] owing many common features with multidrug resistant (MDR) cancer cells, including high expression of ATP-binding cassette (ABC) transporter proteins.[1] CSCs are also slow-growing, quiescent cells and this feature enables them to avoid therapeutic attack of drugs that target rapidly dividing cells.

The main challenge for efficient chemotherapy is the fact that approved anticancer drugs are designed to kill growing cells but they fail to induce permanent cancer eradication because remaining cancer cells resist therapy. These remaining cells responsible for the development of acquired resistance to a variety of anticancer drugs including targeted therapies are CSCs.[6] A huge amount of data could be gathered regarding mechanisms of resistance developed in cancer cells after application of antimetabolites, DNA damaging, microtubule interacting and new targeted drugs.[7] Many in vitro cancer cell models were established for the evaluation of drug resistance largely by selection process through drug exposure or by transfection of genes for ABC transporters. Some of drug resistant models were obtained by sorting the side-population of cancer cells considered to possess stemness and resistant phenotype.[8] Herein, we tried to evaluate the suitability of these models for studying and targeting cancer stem phenotype.

Characterization of CSCs

According to CSC theory, a single CSC should be able to generate a tumour, but for now, there are no means to purify CSC to the uniform degree. Therefore, the CSCs are characterized by their ability to give rise to serially transplantable tumours in immunodeficient hosts while maintaining the original tumour phenotype.[9] In vitro, the presence

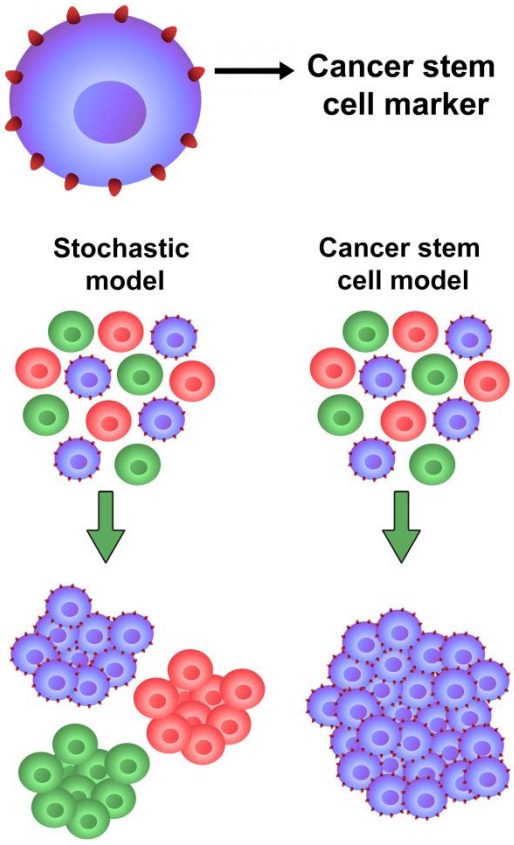

Fig. 1 Stochastic and CSC models of tumour growth

of markers used to isolate CSCs and their capacity to form spheres correlate with their in vivo potential to form tumours in xenografts.[9]

The first direct evidence of the presence of CSCs is provided in 1997, when the authors demonstrated that CD34+CD38- cancer cells from acute myeloid leukaemia (AML) patients could initiate AML in NOD/SCID (non-obese diabetic/severe combined immune deficient) mice.[10] In addition, these cells possessed the ability of self-renewal, proliferation and differentiation. Interestingly, the CD34+CD38- cell surface phenotype of leukaemia stem cells (LSCs) is shared by immature hematopoietic precursors including hematopoietic stem cells (HSCs), implying the possibility that LSCs arise from HSCs. Xenotransplantation followed by serial transplantations - is now widely accepted as a gold standard for identifying CSCs.[2] The similar techniques are established for the characterization of CSCs from solid tumours. The first such report was published in 2003 describing isolation and purification of CD44+CD24- tumourigenic cells from human breast carcinoma by flow cytometry activated sorting (FACS) that were able to form tumours in the mammary pads of NOD/SCID mice. Engrafted tumours exhibited similar morphologic and immunophenotypic heterogeneity to the original specimen cells.[11]

CSC markers

'CSC markers' is commonly used term for proteins that are connected with a particular stem cell phenotype. However, the possibility that CSC markers play an important role in the maintenance of the tumourigenic potential cannot be excluded.[12] CSCs have been frequently isolated based on the expression of more than one cell surface marker for which is known to be expressed in normal stem cells of the tumour originated organ.[13] For example CD133, CD24, CD44, epithelial cell adhesion molecule (EpCAM) and the ATP-binding cassette B5 (ABCB5) have been used to isolate CSCs from breast, brain, pancreas, prostate, lung and ovarian cancers, although the most common are the CD133 and CD44[14] FACS enables the isolation of a side population enriched in CSCs based on the ability of stem cells to efflux fluorescent dyes, such as the Hoechst 33342 or Rhodamine 123. This is due to the overexpression of ABC transporters that are highly promiscuous transporters able to extrude these fluorescent dyes among a variety of their substrates.[15]

CSCs markers have certain limitations. Thus, CSCs marker negative cells can also have tumourigenic and clonogenic properties (Hill RP, 2006). Most experiments suggested that depending on the origin of tumour, the CSCs might be within different phenotypic subpopulations and that different subpopulations can coexist.[13] CD44 is a unique marker, because it has an active role in tumourigenesis and xenograft formation.[16] In a strain of intestinal tumour prone mice, CD44 knockouts had a reduced incidence rate of adenoma.[17] The same was observed in a mouse model of chronic myeloid leukaemia (CML), where BCR-ABL-1 positive progenitors required CD44 for efficient bone marrow homing.[18]

The isolation of CSCs is a challenge, even when working with verified, stably expressed CSC markers. There is relatively small percentage of CSCs in the tumour, so large numbers of cells have to be investigated to acquire enough CSCs for the experiment. CSC markers are still not perfect; not all CSCs express the markers, and some non-CSC cancer cells may also express them. For this reason, the markers can be used to identify CSC-rich subpopulations but might not be able to isolate all of the CSCs existing in the specific type of tumour.

ABC transporters in CSCs

ATP-binding cassette (ABC)-type transporters are membrane transporters that can pump various molecules out of cells by using the free energy of ATP hydrolysis (Fig. 2).[19] CSCs express high levels of specific ABC drug transporters rendering them resistant to chemotherapy. The overexpression of ABC transporters causes the phenomenon referred to as classical multidrug resistance (MDR). By this principal mechanism of MDR, cancer cells develop resistance to various structurally and

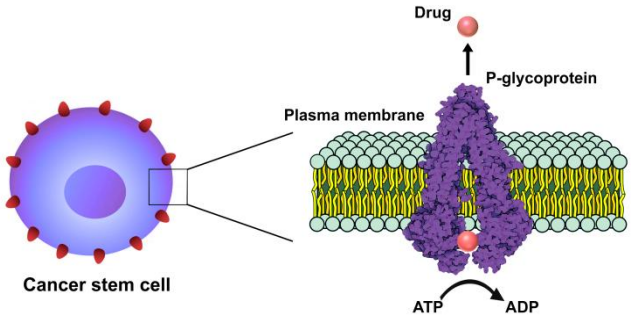

Fig. 2 Expression of P-gp in the membrane of CSC

functionally unrelated drugs.[7] Along with metastatic phenotype, MDR is a leading cause of death by cancer.[20] The P-glycoprotein (P-gp) encoded by the ABCB1 gene was the first ABC transporter identified to be amplified and/or overexpressed in MDR cancer cell lines.[21, 22] This is also the best characterized ABC transporter both biochemically and through mutational analysis.[23] Other two ABC proteins widely overexpressed in cancer cells are ABCC1/MRP1 and ABCG2/BCRP.[24, 25]

P-gp is a 170 kDa glycoprotein which regulates the export of various structurally unrelated anticancer agents from the cell, including paclitaxel, doxorubicin, and vincristine. This protein is normally expressed in tissues that are strategically located to protect against the passage of xenobiotics, including the bronchopulmonary epithelium, hepatobiliary epithelium, renal tubular epithelium, gastrointestinal tract, blood-brain barrier and choroid plexus.[26, 27] As expected, P-gp expression is the highest in tumours derived from tissues that normally express P-gp. However, in many other tumours, the expression of P-gp is induced by chemotherapy.[28]

MRP1 is a 190 kDa protein widely expressed in normal tissues with relatively higher levels in lung, testis, kidney, and peripheral blood mononuclear cells. This transporter has been found to be up-regulated in a variety of solid tumours, including lung, breast, and prostate.[29] Its substrate specificity is broadly similar to that of P-gp. In addition, MRP1 is able to export organic anions, e.g. drugs conjugated to glutathione (GSH), glucuronate, or sulphate.[30]

BCRP, a small protein (70 kDa), known as a half-transporter, which got its name due to the MDR breast cancer cell line co-selected for doxorubicin and verapamil resistance from which it was isolated. It is capable of transporting doxorubicin, mitoxantrone, topotecan, methotrexate, and tyrosine kinase inhibitors, among other substances.[31] This transporter is expressed in a variety of normal tissues with the highest levels found in the placenta, as consistent with the hypothesis of a protective role for the fetus. BCRP protein has been found overexpressed in many MDR tumours.[28]

CSCs are innately resistant to chemotherapy due to slow proliferation rate, enhanced capacity for DNA repair, decreased ability to undergo apoptosis and high ABC transporters expression.[20, 30, 32, 33] These features enable CSCs to survive therapy and relapse, even many years following therapy. CSCs

that survive after chemotherapy enrich the population of chemoresistant cells able to sustain the growth and prograde into a more aggressive and potentially metastatic phenotype.[1] Two models have been proposed to explain the origin of CSC MDR. According to the first model, after exposure to the chemotherapeutic agent, only the CSCs expressing ABC transporters are protected and able to recolonize. The second model suggests that after chemotherapy, only CSCs survive and acquire drug resistance under the pressure of mutations, thus originating new and more aggressive drug-resistant phenotypes.[34]

The identification of ABC gene expression in CSCs has been used to isolate or characterize the CSCs. Methodology of cell sorting according to the drug efflux property of CSCs was employed to isolate CSCs from tumour samples and cancer cell lines.[35, 36] Most cells accumulate fluorescent dyes such as Hoechst 33342 and Rhodamine 123, but a small subset of cancer cells termed side population (SP) efficiently extrudes these fluorescent substrates for ABC transporters.[37] SP cells that correspond to CSCs were identified in neuroblastoma samples as well as in neuroblastoma, breast cancer, lung cancer, and glioblastoma cell lines.[38] However, significant limitation for this CSCs isolation approach is that SP compartment could be composed of stem and non-stem cells, and not all stem cells could be found in the SP fraction.[39]

Signalling pathways controlling CSCs and MDR

Molecular mechanisms which regulate the development of CSCs are still unexplored. Various signalling pathways have been suggested, and some of them are connected with MDR phenotype of cancer cells (Fig. 3).

Hedgehog (Hh) signalling pathway

The Hedgehog (Hh) signalling pathway is crucial for the growth and patterning during embryonic development.[40] Hh pathway is highly conserved across species and important for the expansion and contraction of stem cell numbers during the early stages of embryonal development.[41] In adult organisms, it is involved in different processes related to tissue maintenance and regeneration – proliferation, apoptosis, chromatin modelling and stem cells renewal.[42]

Binding of Hh to the transmembrane receptor Ptch1 initiates signalling via the Hh pathway. Ptch1 inhibits the receptor Smoothened (Smo) by preventing its localization to the primary cilium, a non-motile projection present on most vertebrate cells. Hh is released from the adjacent cell and binds to Ptch1, allowing Smo receptor activation.[43] Gli1/2 transcription factors are released from the Smo receptor complex and translocate to the nucleus, leading to transcriptional activation of Hh associated genes.[44]

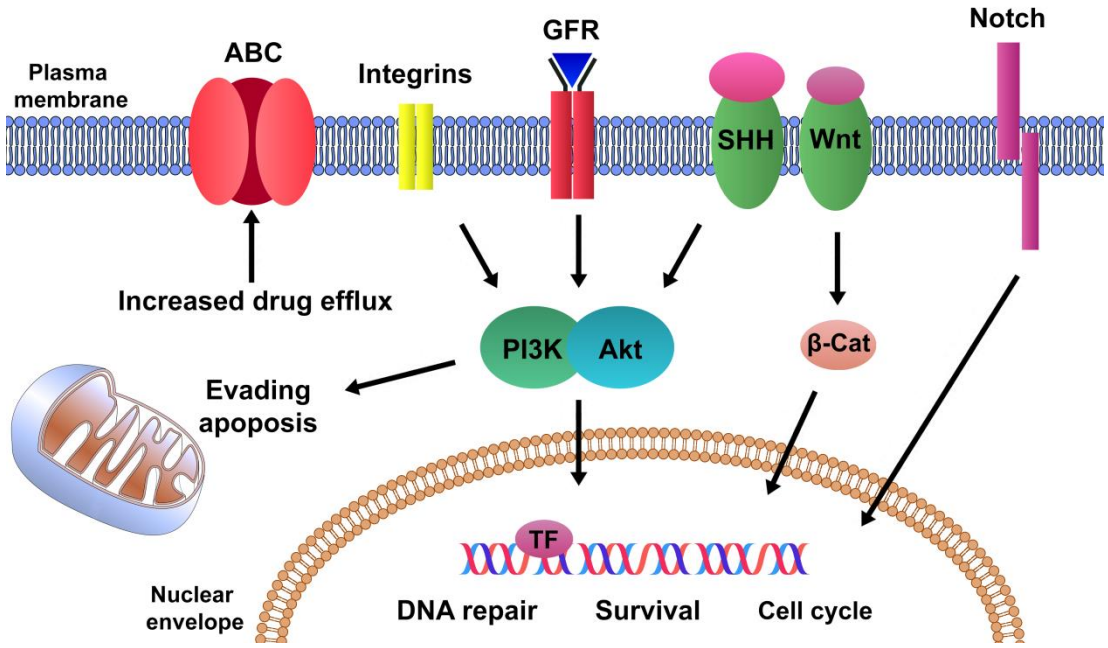

Fig. 3 Molecular mechanisms involved in both development of MDR and maintenance of stemness ABC = ATP-Binding Cassette Transporter; GFR = Growth Factor Receptor; SHH = Sonic Hedgehog; β-Cat = β-catenin; TF = Transcription Factor

Hyperactivation of this pathway, by either mutation or deregulation, has been recognized to cause tumourigenesis in a wide variety of tissues. Hh signalling may also have a key role in maintenance of CSCs and inherence of cancer resistance.[45] Previous studies have suggested that the Hh pathway is essential for the maintenance of CSCs in various human cancer types including pancreatic cancer, gastric cancer and colorectal cancer. It is also recognized as a target for the treatment of cancer MDR. Thus, inhibitors that obstruct the Hh signalling pathway may cause several effects: depletion of CSCs, overcoming MDR, and enhancing the therapeutic effect.[6] These authors also suggested that therapeutics targeting the Hh pathway might improve the outcome of patients with pancreatic cancer by eliminating CSCs.

New therapeutic agents have been developed that target Hh and Smo activation and downstream proteins, such as Gli transcription factors. The first prototype of Hh pathway specific inhibitors is cyclopamine (11-deoxojervine), a plant derived steroidal alkaloid that binds to and inactivates Smo.[46] Cyclopamine was shown to inhibit epithelial-to-mesenchymal transition (EMT) and metastases in pancreatic cancer cell lines.[47] Cyclopamine can act synergistically with gemcitabine to reduce the population of pancreatic CSCs.[47] Similarly, the combination of cyclopamine and temozolomide can reduce the cell mass of glioma CSCs in vivo.[48]

Other synthetic small molecules that are potent inhibitors of Smo are in preclinical investigation.[49, 50] Small synthetic molecules Hh protein inhibitors (HPis) have been also described. Thus, HPi1 inhibits Gli1/2 activation; HPi2 and HPi3 inhibit Gli2 activation while HPi4 inhibits formation of cilia, thus inhibiting Smo activation.[51]

Wnt signalling pathway

The Wnt signalling pathway is another developmental pathway involved in multiple biological processes including embryogenesis, development, cell proliferation, survival and differentiation. The Wnt signalling pathway has been conserved throughout evolution and plays important roles in adult life to maintain homeostasis of tissues by regulating somatic stem cells and their niches.[52-54]

The activity of the Wnt signalling pathway is dependent on the amount of β-catenin in the cytoplasm. Normally, cytoplasmic β-catenin is maintained at a low level through ubiquitin-proteasome mediated degradation, which is regulated by a multiprotein destruction complex containing axin, adenomatous polyposis coli (APC), GSK-3β and casein kinase (CKI). Upon binding of Wnt proteins to a receptor complex Fz/LRP, a protein downstream of the receptor complex is phosphorylated thereby inhibiting GSK-3β, resulting in the accumulation of β-catenin in the cytoplasm. β-catenin avoids degradation and translocates into the nucleus, where β-catenin interacts with members of the T-cell factor (TCF)/ lymphocyte enhancer factor (LEF) family of transcription factors.[55] To generate a transcriptionally active complex, β-catenin recruits the transcriptional coactivators, cAMP response element-binding protein (CBP) or its closely related homolog, p300,[56, 57] leading to the expression of Wnt targeted genes.

Considering the importance of the Wnt pathway in stem cell biology, it is not surprising that aberrant Wnt signalling has been associated with CSCs maintenance. Many of direct Wnt target genes (including LGR5/GPR49, CD44, CD24, CD133, ABC cassette genes, and EpCAM) are also CSC markers. [54] The role of Wnt signalling in glioblastoma stem cells has been recently described,[58] while previous studies have suggested its deregulation in leukemic stem cells when compared with normal hematopoietic stem cells.[59] In leukemic stem cells, upregulation of genes encoding the axin and APC are frequently observed contributing AML genesis.[60]

Activation of Wnt pathway also leads to the upregulation of transcriptional factors that drive EMT. Thus, Wnt upregulates Twist, a key transcriptional factor of EMT,[61] thereby favouring EMT-like processes in breast cancer cells.[62] E-cadherin, a membrane bound glycoprotein involved in the adherence of adjacent cells, may anchor and sequester β-catenin in the membrane thereby preventing its activation. The loss of E-Cadherin during EMT leads to activation of β-catenin resulting in the expansion of the CD44+CD24- subpopulation with CSC-like phenotype.[63]

Wnt/β-catenin signalling pathway appears to play an important role in ABCB1/MDR-1 transcription and thus P-gp expression. Importantly, **m**ultiple TCF binding elements were identified in the ABCB1 promoter (-1813 to -275 bp). Since the P-gp encoded by the ABCB1 gene is responsible for chemoresistance of different tumour types, it is obvious that Wnt pathway is involved in the development of MDR phenotype in cancer cells.[7]

Accumulated evidences suggest that the aberrant Wnt signalling pathway may cause cancer development.[64] Therefore, Wnt pathway represents an important target for new anticancer strategies.

Inhibitors of the Wnt signalling pathway can be grouped into two classes: small-molecule inhibitors and biologic inhibitors. Small-molecule inhibitors include existing drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) and molecular-targeted agents such as the CBP/β-catenin antagonist ICG-001. Biologic inhibitors include antibodies, RNA interference (RNAi), and recombinant proteins. The majority of these inhibitors are in the preclinical stage of development.[65]

NSAIDs, such as aspirin, inhibit the activity of cyclooxygenase (COX), a key enzyme in the arachidonic acid cascade. A number of experimental and epidemiological studies suggested that aspirin and other NSAIDs show chemopreventive effects mainly against colon cancer,[66-68] and inhibition of the Wnt signalling pathway was indicated as one of their potential mechanisms of action.[69] For instance, increased COX-generated prostaglandin E2 suppresses β-catenin degradation, resulting in activation of Wnt signalling. Therefore, suppression of elevated COX activity in cancer cells is likely to be an important factor for the anticancer activity of NSAIDs. On the other side, treatment of colon cancer cell lines with celecoxib, a COX-2 selective inhibitor suppressed Wnt signalling by inducing the degradation of TCFs, independently of COX-2 inhibition.[70, 71]

The small molecule ICG-001 selectively inhibits Wnt signalling by interrupting β-catenin binding to the transcriptional coactivator CBP.[72] ICG-001 treatment of colon cancer cell lines resulted in apoptosis, while sparing normal colon epithelial cells. ICG-001 showed high selectivity towards cancer cells without interacting with the highly homologous coactivator p300.[72] CBP/β-catenin-mediated transcription is essential for stem and/or progenitor cell maintenance and proliferation, whereas a switch to p300/β-catenin mediated transcription is the critical step to initiate differentiation.[73] Therefore, ICG-001 seems to initiate the key switch from β-catenin and CBP interaction to β-catenin and p300 interaction, resulting in initiation of cell differentiation.[71]

Therapeutic monoclonal antibodies against Wnt-1 and Wnt-2 have been developed and shown to inhibit Wnt signalling and suppress tumour growth in vivo.[74, 75] Similarly, small interfering RNA (siRNA) against Wnt-1 and/or Wnt-2 had potential therapeutic utility in cancer cell lines.[76]

Notch signalling pathway

Notch signalling has a critical role in regulating cell-to-cell communication during embryogenesis, cellular proliferation, differentiation, and apoptosis.[77] Mammalian membrane bound Notch ligands interact with transmembrane Notch receptors (Notch 1–4). The pairing of Notch ligand-receptor results in coordinated communication between adjacent cells.[78] Once ligand-receptor binding occurs, the Notch receptor undergoes a conformational change to expose a previously protected site to proteolytic cleavage by metalloprotease and γ-secretase.[79] These catalytic steps cleave the intracellular and membrane domain and release the active Notch intracellular domain (NICD) into the cytoplasm. NICD undergoes nuclear translocation and modulate Notch-specific gene expression.

Inappropriate Notch activation stimulates proliferation, restricts differentiation, and/or prevents apoptosis. Notch functions as an oncogenic protein in most human cancers including cervical, lung, colon, head and neck, prostate and pancreatic cancer, while it acts as a tumour suppressor in skin cancer, hepatocellular carcinoma and small cell lung cancer.[80] The strongest evidence to date for a role of Notch in CSCs is in breast cancer,[81] embryonal brain tumours,[82] and gliomas.[83, 84] Additionally, there are evidences that Notch signalling might contribute to cancer metastasis.[85] Notch pathway plays a critical role in the linkages between angiogenesis and CSCs self-renewal and is thus receiving increased attention as a target to eliminate CSCs.[86] It is showed that the self-replication and tumour formation capacity of leukemic CSCs is reduced by blocking Notch signalling activation.[87] Recently, it was discovered that Notch1 signalling interfere with the development of MDR phenotype by promoting chemoresistance through regulation of MRP1 expression in prostate CSCs.[88] Therefore, targeting Notch signal transduction pathway may be good therapeutic strategy for both treatment of cancer MDR and elimination of CSCs.

Inhibition of γ-secretase mediated Notch cleavage is a primary focus for the development of targeted therapeutics. Several pharmaceutical companies have developed γ-secretase inhibitors (GSIs) that are in the early clinical development.[89] GSIs abolish the formation of secondary mammospheres (an indicator of stem-like cells) from a variety of human breast cancer cell lines as well as primary patient samples.[90] In situ ability of breast ductal carcinoma to form mammospheres is dramatically decreased by GSIs and Notch 4 monoclonal antibody.[81] Inhibition of Notch 1 with monoclonal antibodies significantly reduced the CD44+CD24−/low subpopulation and lowered the incidence of brain metastases from a breast cancer cell line.[91] In addition, Notch inhibition selectively depleted medulloblastoma CSCs.[82] The same was observed in glioblastoma CSCs.[83] Importantly, Notch seems to confer radio-resistance to glioma CSCs. GSI treatment selectively enhanced radiation-induced death of glioma CSCs but not bulk glioma cells. This effect was also accomplished by Notch 1 or Notch 2 knockdown and accompanied by AKT inhibition.[84]

In addition, NOTCH1 could bind to ABCC1 promoter region and regulate MRP1 protein expression. Thus, it was shown that inhibition of activated form of Nothc1 (ICN1) with shRNA enhanced prostate CSCs chemosensitivity due to the decrease in MRP1 expression.[92] Recently, it was described that cisplatin-induced DNA damage enriched CD133+ cells in NSCLC in vitro and in vivo through Notch signalling.[93] Elevation of CD133+ cells, which were also positive for P-gp and BCRP, induced cross-resistance to functionally unrelated drug – paclitaxel, thus confirming the connection of Notch signalling with the development of cancer MDR.

RAS/MAPK and PI3K/AKT pathway

Deregulated signalling through the RAS/MAPK and PI3K/AKT pathways is often the result of genetic alterations in critical components in these pathways or upstream activators (growth factor receptors and integrins). Unrestricted cellular proliferation and decreased sensitivity to apoptotic-inducing agents are typically associated with activation of these pro-survival pathways.[94] The RAS/MAPK and PI3K/AKT signalling pathways consist of kinases cascades that are regulated by phosphorylation and de-phosphorylation by specific kinases, phosphatases as well as GTP/GDP exchange proteins, adaptor proteins and scaffolding proteins.[95] Mutations often occur in the genes encoding pathway constituents (e.g., RAS, RAF, PIK3CA, PTEN, AKT, TSC1, TSC2) or in upstream receptors which activate these pathways.[96]

Different roles of the RAS/MAPK and PI3K/AKT pathways have been identified including development of stemness phenotype, senescence, aging and sensitivity to targeted therapy.[97-99] CSCs have unique properties as they can be both quiescent and resistant to chemotherapeutics and hormonal based drugs.[97] It is observed that some drug resistant breast cancer cells with properties similar to CSCs display elevated activation of the RAS/MAPK and PI3K/AKT signalling cascades.[100, 101] Some data suggests these CSCs are more sensitive to MEK and mTOR inhibitors than either the parental or drug resistant cells from which they were derived.[100] Therefore, targeting the RAS/MAPK and PI3K/AKT pathways could be very important in terms of CSCs elimination.

Quiescence and cell cycle entry are tightly controlled in non-cancer stem cells (NSCs). This facilitates self-renewal and prevents depletion of the stem cell pool. One of the key signalling pathways that control cell cycle appears to be the PI3K/AKT pathway. Activation of PI3K, or inactivation of PTEN, a tumour suppressor that attenuates PI3K/AKT signalling pathway, leads to downstream activation of mTOR through AKT. mTOR inhibitors such as rapamycin and its derivatives demonstrated strong efficacy against AML cell lines and AML patient samples.[102, 103] These studies not only showed a decreased survival of AML blasts due to the induction of apoptosis[102] but also demonstrated a loss of colony-forming potential of AML blasts while sparing normal hematopoietic progenitors.[103] This supports the suggestion that rapamycin targets AML CSC self-

renewal, although sparing self-renewal of normal HSCs. Furthermore, rapamycin led to eradication of leukaemia-initiating cells arising due to PTEN deletion in mice and further restored NSCs function, which was impaired through disruption of PTEN.[98] Taken together, these data suggest that mTOR inhibitors may target both self-renewal and survival mechanisms in cancer.[103-105]

Recent findings demonstrated that targeting either RAS/MAPK or PI3K/AKT pathway may chemosensitize anaplastic thyroid carcinoma cells. Inhibition of RAS/MAPK signalling was more effective in combination with paclitaxel, whereas inhibition of PI3K/AKT was more effective in combination with doxorubicin. The suppression of downstream effector of both pathways mTOR equally synergized with both chemotherapeutics.[106]

TGF-β pathway

Transforming growth factor-β (TGF-β) provides important regulatory signals during embryonic development and tissue homeostasis in adults.[107] At the cellular level, TGF-β controls several biological events such as cell cycle, apoptosis and EMT.[108] At the tissue level, TGF-β regulates the differentiation and immunological response of B and T lymphocyte as well as tissue interactions important during embryonic organogenesis.[109] TGF-β binds to a type II receptor, which constitutively recruits and phosphorylates a type I receptor. The type I receptor subsequently phosphorylates and activates a number of downstream effector proteins, most notably the Smad transcription factors family.[110] The Smad complexes can associate with accompanying transcription factors to activate expression of target genes that cause changes in cellular differentiation. Notably, Smads associate with Zeb proteins to repress expression of E-cadherin during the initiation of EMT.[111]

Abnormalities in the TGF-β signalling relate to the development of multiple cancer types including breast, colon, liver and lung.[112-115] However, potential role of TGF-β in CSCs only recently emerged. TGF-β signalling is involved in the maintenance and function of breast, liver, lung and glioblastoma CSCs.[116-119] TGF-β upregulates the expression of CD133 in hepatocellular carcinoma through Smad-dependent transcriptional mechanism.[117] In addition, TGF-β selectively induces self-renewal of the glioma-initiating cells by two independent pathways. First pathway includes Smad-dependent induction of leukaemia inhibitory factor and the sequential activation of the JAK-STAT pathway leading to the increase in neurosphere formation and prevention of neurosphere differentiation.[119] Second pathway involves expression of Sox4, which binds to Oct4, and this complex cooperatively activates the enhancer of Sox2.[120, 121]

Molecular mechanisms that regulate EMT and invasiveness could also co-regulate ABC transporter expression. TGF-β signalling pathway has an established role in promoting EMT by down-regulating E-cadherin through a number of transcription factors, such as Twist, Snail and Zeb1.[122] Saxena et al. demonstrated that TGF-β treatment of MCF-7 cell line induce the expression of five ABC

transporters.[123] The promoters of ABC transporters carry several binding sites for EMT-inducing transcription factors and overexpression of Twist and Snail increases the promoter activity of ABC transporters. In contrast, application of TGF-β decreased the levels of BCRP expression in gastric carcinoma cells and eliminated cancer-initiating cell population.[124]

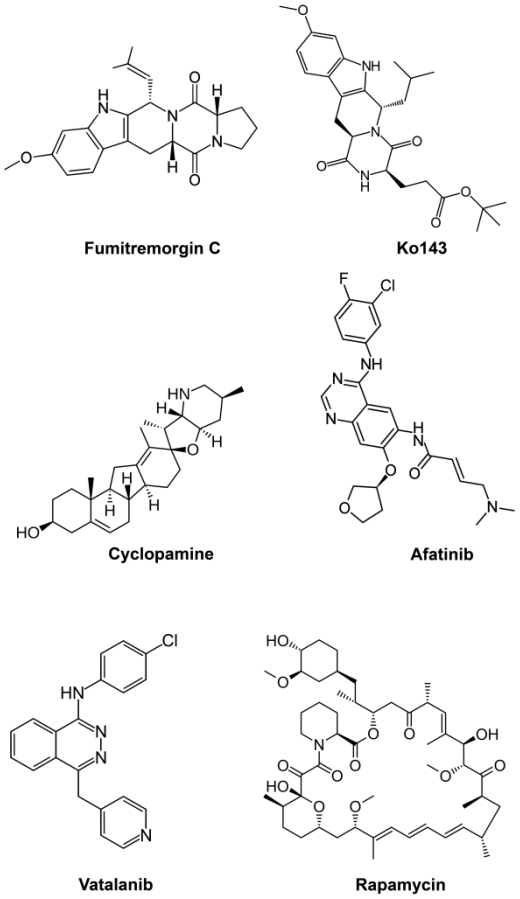

Fig. 4 Targeting CSCs and MDR – representative inhibitors **Fumitremorgin C** - a natural product that inhibits BCRP; **Ko143** – a synthetic derivative of Fumitremorgin C; **Cyclopamine** – a plant derived steroidal alkaloid which inhibits Hh pathway; **Afatinib** - a TKI, which targets EGFR, HER2, and HER4; **Vatalanib** - a TKI, which targets VEGFR1, -2, and -3, PDGFR, c-kit and suppresses the function of P-gp and BCRP; **Rapamycin** - an mTOR inhibitor**.**

Targeting ABC transporters

Investigators have designed numerous strategies to evade, neutralize or even exploit ABC efflux pumps to overcome drug resistance. Finding new modalities for inhibition of these transporters is a

main stream in re-establishment of drug sensitivity and improvement of the drug effectiveness in cancer therapy.[125-127]

Three generations of compounds have been developed to modulate the activity of ABC transporters. First-generation of ABC inhibitors are compounds that are developed to treat other conditions: verapamil (a calcium channel blocker used as an antihypertensive), quinine (an antimalarial drug) and cyclosporine A (an immunosuppressant). These inhibitors had low binding affinity and numerous adverse side effects such as dose-limiting toxicity and cardiac toxicity in the case of verapamil.[128, 129]

In the development of second-generation of ABC inhibitors, efforts were focused on increasing the specificity for P-gp, while decreasing toxicities. Valspodar (PSC-833), a derivative of cyclosporine A, is a representative of second-generation inhibitors. It showed higher potency and no immunosuppressive side effects in comparison with cyclosporine A and other first-generation inhibitors.[130] Another second-generation inhibitor Biricodar (VX-710) was shown to be potent modulator of both P-gp and MRP1 in vitro.[131, 132] However, clinical trials with these inhibitors failed in some cases because of pharmacokinetic interaction between the chemotherapeutic agent and the ABC inhibitor.[133, 134]

Therefore, the next, third, generation of inhibitors were designed to be more potent and without undesired side effects. Tariquidar (an anthranilamide, XR9576), Elacridar (an acridone caroxamide) and Zosuquidar (LY335979, quinolone derivative) are representatives of this group of inhibitors. Generally, third-generation of P-gp inhibitors are less toxic, do not interfere with pharmacokinetics of anti-cancer drugs and have better outcomes in clinical trials than first- and second generation of inhibitors.[135, 136]

ABC inhibitors might be considered as CSCs sensitizing agents that target the most crucial and most resistant cells in cancer. However, the most studies have shown that CSCs preferentially overexpress BCRP, rather than P-gp, which inhibition has been evaluated in most clinical studies.[38] Therefore, there is a necessity for development of new inhibitors specific for BCRP transporter. Particularly promising source of new P-gp and BCRP inhibitors are natural compounds and their derivatives. The fourth generation of ABC transporters' inhibitors mainly comprises of these compounds.

Successful inhibition of ABC transporters on expressional and functional level with various approaches using MDR cancer cell lines is summarized in Table 1.

It was shown that dofequidar fumarate, an orally active quinoline compound, greatly reduced the cell number in CS-like SP cells isolated from various cancer cell lines. It inhibited the efflux of

chemotherapeutic drugs and increased the sensitivity of SP cells to anticancer drugs. The in vitro vesicle transporter assay clarified that dofequidar has the ability to suppress BCRP function.[137]

The compound fumitremorgin C (FTC) is a natural product found to specifically inhibit BCRP (Fig. 4).[138] However, this compound is toxic to cells, as well as to mice, and is not suitable for clinical studies. Chemically synthesized derivatives of FTC such as Ko143 have been developed, and several of these showed high specificity and low toxicity (Fig. 4).[139] Interestingly the compound GF120918, which is P-gp inhibitor, also shows activity against BCRP.[140]

Another class of natural products - jatrophane diterpenoids were shown to be potent inhibitors of P-gp. Their advantage is selectivity towards cancer cells and the capacity to sensitize MDR cancer cells of different origin (non-small cell lung carcinoma – NSCLC, colorectal carcinoma and glioblastoma) to conventional chemotherapeutics.[141-144]

Studies of pan-ABC inhibitors found that peptides mimicking transmembrane domains of ABC transporters could be designed as selective and specific inhibitors for any of these transporters.[145]

Another investigation showed that NK-lysin derived cationic peptide NK-2 discriminates and preferentially kills P-gp overexpressing cancer cells in NSCLC and colorectal carcinoma cell lines. Acting in a unique way, NK-2 peptide eliminates the P-gp high-expressing cells from heterogeneous cancer cell population likely making CSCs more vulnerable to chemotherapy.[146]

An alternative strategy to functional inhibition of ABC transporters in CSCs is the attempt to regulate the protein expression levels of these transporters. It has been indicated that Hedgehog (Hh) signalling can regulate the expression of both P-gp and BCRP. Treatment of PC3 cells with cyclopamine (an Hh pathway specific inhibitor) downregulated the expression levels of P-gp and BCRP (Fig. 4).[147] In addition, it was recently found that abnormal expression of the Hh signalling pathway transcription factor Gli1 is involved in the regulation of P-gp and BCRP in ovarian cancer. Inhibition of Gli1 expression was able to decrease P-gp and BCRP gene expression levels and enhance the response of ovarian cancer cells to specific chemotherapeutics.[148]

The regulatory role of miR-125b was confirmed in SP cells of breast cancer with high BCRP and P-gp expression and chemoresistant phenotype. Namely, antisense oligonucleotides for miR-125b decreased the ability of breast SP cells for colony formation.[149]

In human hepatocellular carcinoma (HCC) upregulation of BCRP enhanced the capacity of proliferation, doxorubicin resistance, migration, and invasion potential, while its downregulation significantly decreased these malignant behaviours in tissues and cell lines.[150] An antibiotic, the N-linked glycosylation inhibitor tunicamycin was able to dramatically reduce BCRP expression, alter its subcellular localization, and reverse its drug efflux effect in multiple HCC cell lines.[151]

Liu PP et al. showed that glucose upregulates the SP fraction through ATP-mediated suppression of AMPK and activation of the AKT pathway, leading to elevated expression of the ATP-dependent efflux pump BCRP. Therefore, inhibition of glycolysis by 3-BrOP significantly reduced cancer SP cells' fraction in vitro and impaired their ability to form tumours in vivo.[152]

Overcoming MDR and eliminating CSCs

CSC populations are more resistant to conventional cancer therapies than non-CSC populations. Thus, the key feature to characterize SP cells as CS-like cells is higher resistance to chemotherapy and presence of principal mediators of MDR – ABC transporters. In the absence of specific CSCs surface markers, only valuable purification strategy to obtain CSCs is sorting of SP according to the activity of ABC transporters (Hoechst 33342 and Rhodamine 123 accumulation assays). The accumulation of a Hoechst dye 33342 showed that human NSCLC cell lines consist of 0.03 - 6.1% cells which represent SP with increased expression of BCRP.[32] These potential CSCs obtained by FACS demonstrated tumourigenicity in mice resistant to various chemotherapeutic agents.[153] Other authors showed that SP cells obtained from esophageal cancer really mirror CSCs, contribute to the resistance to 5-FU and cisplatin, and regulate EMT.[154]

Moreover, the increased expression of stemness markers was observed in patients who received first line chemotherapy.[155] This implies that CSC fraction could be enriched after chemotherapy and responsible for the development of acquired resistance. Ovarian cancer SP cells are more resistant to chemotherapeutic drugs than non-SP cells. BCRP has been accepted as an ABC transporter characteristic for ovarian cancer SP.[165] However, one study demonstrated that actually P-gp facilitates drug resistance in ovarian cancer SP in a response to paclitaxel treatment.[156] Inhibition of P-gp expression restored the sensitivity to paclitaxel and enabled the elimination of ovarian cancer cells, including SP cells.

We also demonstrated that SP successive fractions (named Rho -, Rho - -, Fig. 5A) of anaplastic thyroid carcinoma cell line 8505C expressed P-gp and BCRP transporters (Fig. 5B), which enabled the exclusion of the Rhodamine 123 dye from potential CSCs (Fig. 5B, C). The expression of these transporters is a prerequisite to isolate CSCs by FACS as SP fraction and at the same time the reason for resistance to chemotherapy. The percentage of CSCs in 8505C was low at only 1.6% (Fig. 5A) which is in accordance with previous reports of CSC in anaplastic thyroid carcinoma cell lines.[166, 167] We found that Rho- cells are significantly more resistant to paclitaxel than their parental 8505C cells (Fig. 5B).

Herein, we review different strategies investigated on MDR cancer cell lines able to eradicate CSCs and overcome MDR (Table 1).

Several representatives of tyrosine kinase inhibitors (TKIs) gefitinib, imatinib, and lapatinib showed the potential to interact with BCRP by inhibiting its function and consequently enhancing the efficacy of classic chemotherapeutics.[168]

Afatinib (BIBW 2992), a TKI, which targets ErbB family members EGFR, HER2, and HER4, exhibited stronger activity in lung cancer patients that harbor the gefitinib/erlotinib-resistant mutant EGFR.[169] Interestingly, afatinib was able to eliminate CS-like SP cells and inhibit their self-renewal ability in vitro and in vivo by exerting unique mechanism of BCRP promoter methylation (Fig. 4).[157] By this means, afatinib significantly enhanced the efficacy of chemotherapeutic agents. These findings are important because there are indices that positive immunostaining for BCRP could be considered as a prognostic factor of shorter survival in patients with advanced NSCLC.[170]

Another study with an orally active small molecule multi-TKI vatalanib (PTK787/ZK22584) showed its potential to sensitize MDR colon cancer cells to conventional chemotherapeutics. Vatalanib inhibits all known VEGFRs (VEGFR1, -2, and -3), PDGFR, and stem-cell factor receptor c-kit[171] but also suppresses the function of P-gp and BCRP thus providing efficient eliminating of colon CSCs (Fig. 4).[158] A specific TKI c-Met inhibitor SU11274 increased the chemosensitivity of gastric carcinoma CSCs with high expression levels of BCRP and P-gp to the irinotecan treatment.[159]

Besides increased drug efflux driven by ABC transporters, mechanisms of MDR comprise, pharmacokinetic alterations, tumour micro-environmental changes, slow progression through cell cycle, drug inactivation by detoxification, drug target modification and evading apoptosis.[172]

Quiescence of MDR cells is also a physiological property of CSCs that serves as a shield against harmful insults. It was hypothesized that CSCs contribute to tumour dormancy.[173] However, specific stimulation from the microenvironment can promote their growth.[174] Therefore, maintaining the CSCs in a quiescent state by

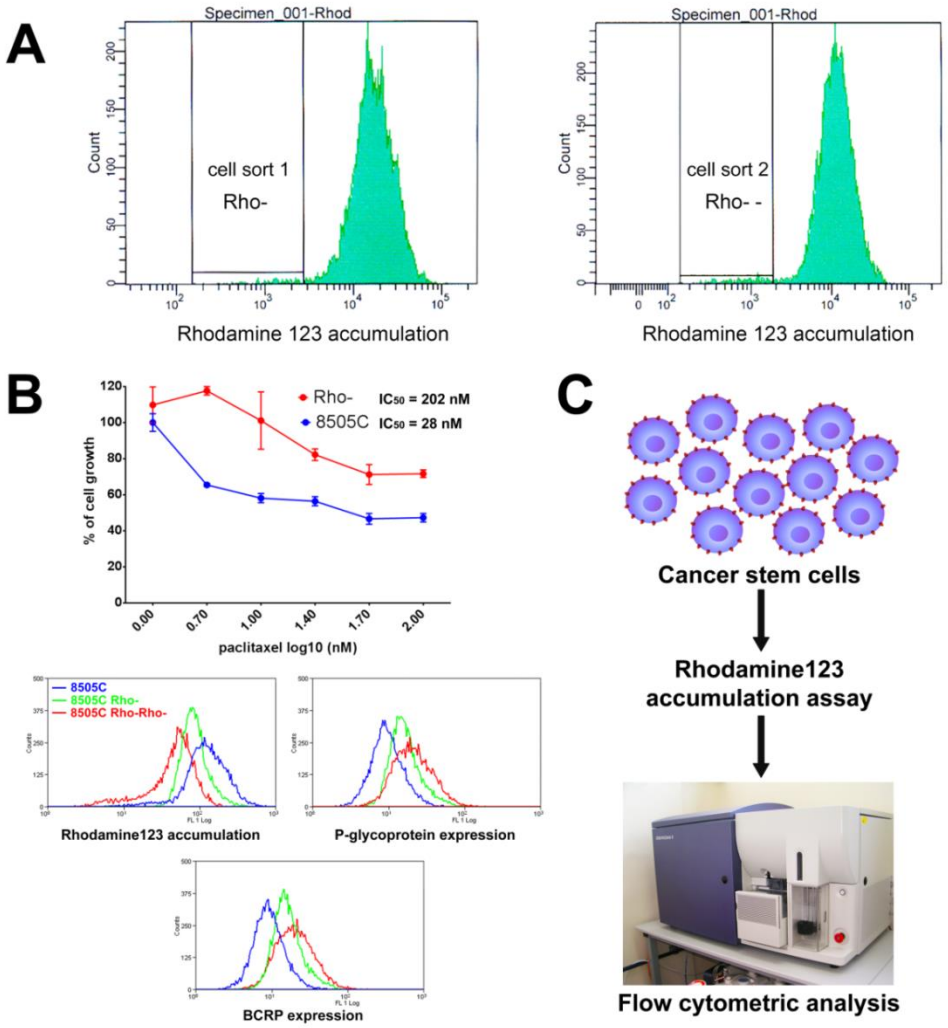

Fig. 5 Successive sorting of anaplastic thyroid carcinoma SP cell fractions by Rhodamine 123 accumulation assay (A) Flow cytometric profiles of Rhodamine 123 negative cells that were sorted (sort 1 and sort 2); (B) Sorted Rho - and resorted Rho - - cells displayed increased P-gp and BCRP expression and resistance to paclitaxel; (C) Illustration how potential CSCs could be obtained from SP sorting.

Table 1 Exploitation of MDR cancer cell line models aimed to find more efficient approaches for overcoming MDR and eliminating CSCs

Type of cancer cell line	Resistance model	Treatment strategy	Functional inhibition of ABC transporters	Expression inhibition of ABC transporters	Reference
HeLa-human cervix carcinoma, KB-3-1-human epidermoid carcinoma, K562-human chronic myeloid leukemia, BSY-1, HBC-4, and HBC-5-human breast cancer, U251-human glioma, Capan-1-human pancreatic cancer, KM12-human colon cancer, MKN74-human stomach cancer	SP cell sorting	dofequidar fumarate- quinoline compound	BCRP		[137]
S1-M1-3.2-colon cancer	Mitoxantrone selection	fumitremorgin C-natural product	BCRP		[138]
MEF3.8/Bcrp1 A2, 77.1/MDR1 clone 5, NIH3T3-mouse fibroblast cell lines; 2008/MRP1 clone 4, 2008/MRP3 clone 8-human ovarian cancer, MDCKII/MRP2	Transfection and/or SP cell sorting	Ko143-fumitremorgin C derivative	BCRP		[139]

clone 17-human epithelial cells, HEK293/MRP4 clone 4.1, HEK293/MRP5 clone 5 I-human embryonic kidney cells, IGROV1, IGROV1T8-human ovarian carcinoma, MCF7-human breast cancer, CCRF-CEM-Human T cell lymphoblast-like cells, WiDr-human colon adenocarcinoma, A549-human non-small cell lung carcinoma				
NCI-H460/R-human non-small cell lung carcinoma, DLD1-TxR-human colorectal adenocarcinoma, U87-TxR -human glioma	Doxorubicin/paclitaxel selection	Jatrophane diterpenoids-natural products	P-gp	[141-144]
NCI-H460/R-human non-small cell lung carcinoma, DLD1-TxR-human colorectal adenocarcinoma	Doxorubicin/paclitaxel selection	NK-2-antimicrobial cationic peptide	P-gp	[146]

SEG-1-human esophageal adenocarcinoma, LnCaP, PC3-human prostate carcinoma, DM14-human metastatic squamous cell carcinoma	Intrinsic resistance	cyclopamine	BCRP, P-gp	[147]
IOSE398-human ovarian epithelial cells, OVCAR3, OVCAR5, OVCAR4, OVCAR8, SKOV3, ES-2, IGROV1, and A2780-human ovarian cancer	Intrinsic resistance	siRNA for Gli1	BCRP, P-gp	[148]
MCF-7, T47D, MDA-MB-231-human breast cancer and primary cancer cells from breast cancer patients	SP cell sorting	antisense oligonucleotides for miR-125b	BCRP and P-gp	[149]
SMMC-7721-human hepatocellular carcinoma	SP cell sorting	siRNA for BCRP	BCRP	[150]
Huh7, PLC/PRF/5, HEK 293T, SMMC-7721, MHCC-97L, MHCC-97H, MHCC-LM3-human	SP cell sorting	tunicamycin	BCRP	[151]

hepatocellular carcioma and HCC-LY5-primary hepatocellular carcinoma cell line					
A549, NCI-H460-human non-small cell lung carcinoma and LoVo-colon cancer	SP cell sorting	3-BrOP- glycolysis inhibitor		BCRP	[152]
IGROV1, HeyA8, HeyA8MDR-ovarian cancer	SP cell sorting	P-gp silencing by morpholino oligonucleotide	P-gp		[156]
S1-MI-80-human colon carcinoma, MCF7/FLV1000-breast carcinoma, CNE2, CNE-2s-18-nasopharyngeal carcinoma	Mitoxantrone and flavopiridol selection	Afatinib (BIBW 2992)		BCRP	[157]
S1M1 80-human colon cancer	BCRP transfection	vatalanib (PTK787/ZK22584)	BCRP, P-gp		[158]
OCUM-2M, OCUM-2D, OCUM-2MD3-human gastric cancer	SP cell sorting	c-Met inhibitor SU11274		BCRP and P-gp	[159]
NCI-H460/R, COR-L23-human non-small cell lung carcinoma	Doxorubicin selection and intrinsic resistance	CXCR4 inhibitor (WZ811)		P-gp, MRP1	[160]
U251MG Dox-R, U373MG Dox-R-human glioma	Doxorubicin selection	Silencing and inhibition of CD133, PI3K,		P-gp	[161]

cells		DNA-PK and AKT		
HeLa-human cervical cancer, Saos-2-human osteosarcoma, Hep3B, PLC, and 97L (97H)-human hepatocellular carcinoma and MIHA-normal hepatic cells	Cisplatin, doxorubicin and 5-fluorouracil selection	Silencing of Oct4	BCRP	[162]
BxPc-3, PANC-1, SW1990-human pancreatic cancer	SP cell sorting	multiple targeting of Sox2/Oct4/c-Myc	BCRP	[163]
HL60/ABCG2-human acute promyelocytic leukemia and Jurkat/ABCG2	Lentiviral transduction of ABCG2 to cell lines	PI3K inhibitor (LY294002), mTOR inhibitor rapamycin	BCRP	[164]

blocking specific receptors and signalling pathways may inhibit tumour initiation and metastasis.[175] On the other side, inducing dormant CSCs to enter the cell cycle probably can restore radio and chemosensitivity. Cytokines such as interferon-α (IFNα) and granulocyte-colony stimulating factor (G-CSF) can efficiently promote the cycling of normal HSCs as well as LSCs. Therefore, combining IFNα and G-CSF with chemotherapeutic agents may eradicate LSCs.[176]

Cytokine receptor CXCR4, which is involved in tumour metastasis, represents another target for CSCs eradication.[19] New study revealed the association between CXCR4 and P-gp/MRP1 in NSCLC patients. In addition, inhibition of CXCR4 was able to successfully sensitize resistant NSCLC cells to doxorubicin and equally overcome either acquired or intrinsic MDR.[160]

Xi et al. showed that it is possible to sensitize MDR glioblastoma cells characterized with stemness marker CD133 expression and high activity of DNA-dependent protein kinase DNA-PK to doxorubicin treatment after inhibition of PI3K.[161] Moreover, downregulation of CD133 and DNA-PK catalytic subunit, or inhibition of PI3K significantly decreased AKT, NF-κB and P-gp expression in doxorubicin resistant glioblastoma cells. Apparently, targeting both CD133 and DNA-PK in glioblastoma CSCs may increase the efficacy of classic chemotherapeutics.

Oct4 was identified as a potential CSC marker due to its role in the maintenance of pluripotency in embryonic cells[177] and development of cancer MDR.[162, 178] Recent results suggest that Oct4 plays an important role in the survival of oxaliplatin resistant colorectal carcinoma cells enriched for CSCs. Its expression is tightly connected with anti-apoptotic activity of STAT3/Survivin pathway.[179] It was found that Oct4 can mediate chemoresistance in liver cancer through the Oct4-AKT-BCRP pathway.[162] In addition, pancreatic cancer SP cells were found to overexpress stemness marker CD133, pluripotency maintaining factors Nanog, Sox2 and Oct4, oncogenic transcription factor c-Myc, signalling molecule Notch1 and BCRP.[163] Targeting multiple mediators of cancer cells' survival (Sox2/Oct4/c-Myc) led to the chemosensitization of pancreatic cancer SP cells.

Recent findings suggest that targeting PI3K/AKT pathway with its downstream effectors (mTOR, caspases, cell cycle protein family and NF-κB) is beneficial for the eradication of breast CSC population. Thus, PI3K inhibitor BKM120 showed significant potential for both overcoming drug resistance and eliminating CSCs.[180]

Bleau AM at al. reported that the SP phenotype of glioma cells resulted from BCRP activity, which localization to the plasma membrane is mediated by AKT. Furthermore, the loss of PTEN as well as temozolomide application increased the SP fraction of glioma CS-like cells. This implied BCRP regulation through PI3K/AKT pathway.[181]

Similarly, overexpression of BCRP in Jurkat and HL60 cells led to an increased SP fraction, up-regulated levels of phosphorylated-PI3K and phosphorylated-AKT, and enhanced drug resistance, all of which could be attenuated by treatment with either the PI3K inhibitor LY294002 or the mTOR inhibitor rapamycin (Fig. 4).[164]

Since TGF-β signalling plays a significant role in maintenance of CSCs, selective targeting of TGF-β may be considered as an effective therapeutic strategy for the treatment of various cancer types. Current approaches with TGF-β inhibitors, such as type I receptor inhibitor LY2109761, led to the development of acquired chemoresistance in cancer patients.[108] However, a clinical study with the improved type I receptor inhibitor LY2157299 showed strong beneficial effects of anti-TGF-β therapy in glioma patients.[182] Furthermore, other type I receptor inhibitors SB431542 and LY364947 induced differentiation of glioma-initiating cells by downregulation of Sox4 and Sox2 expression.[120] To improve delivery of LY364947, polyethyleneimine/polyethylene glycol-conjugated nanoprticles (NPs) loaded with this TGF-β inhibitor were designed.[183] This approach significantly improved therapeutic efficiency in tumour xenograft models compared to the treatment with free LY364947. Interestingly, gold NPs (AuNPs) may selectively capture TGF-β through S–Au binding between cysteine and disulphide residues resulting in deactivation of TGF-β signalling.[184]

Conclusions

Although CSC concept still remains discussable, chemoresistant cancer cells represent the force that lead to cancer relapse and progression. Current therapeutic strategies fail to eradicate CSCs often inducing the selection of resistant clones. There are many overlapping mechanisms that govern MDR and maintain the stemness phenotype. Among them, ABC transporters' overexpression is probably the most important. Overcoming MDR by inhibition of ABC transporters can sensitize CSCs to conventional chemotherapy. In addition, the evidences imply that targeting the key molecules conferring stemness to CSCs can efficiently suppress the activity of ABC transporters and eliminate CSC-like phenotypes.

All these findings indicate that targeting either MDR or CSCs may lead to the eradication of aggressive phenotypes. Since CSCs share the same or similar cell markers with NSCs, CSC-targeted therapy may produce severe toxicity in vivo, so it is important to find more selective therapeutic approaches. To that end, MDR cancer cell line models could be considered as a valuable tool for the evaluation of new CSC targeted therapies.

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