Novel strategies to target therapy resistant cancer stem cells

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Abstract

Cancer therapies are frequently ineffective in irradicating all malignant cells which often results in tumor recurrence. Intricate genetic, epigenetic, and metabolic differences between cancer subpopulations lead to heterogenous neoplasms. Cancer stem cells (CSCs) are often resistant to conventional chemotherapeutic drugs and are believed to be the most invasive tumor subpopulation. The latter characteristic arises from their epithelial to mesenchymal transition phenotype. These CSCs are thought to be the cause of tumor recurrence. In the past two decades, researchers have identified several different stemness regulatory pathways and specific surface makers that may be exploited for targeting CSCs. Antagonizing stemness pathways to minimize self-renewal properties is an approach that is extensively researched. However, this technique has its drawbacks since these pathways are often also expressed in healthy cells. A novel technique that is currently in its infancy, might be better suited for specifically targeting CSCs. The use of gold nanoparticles (AuNPs) which are highly versatile may be used in different applications. AuNPs can be conjugated with antibodies and toxins to target specific cancer subpopulations in heterogenous tumors. Although finding specific surface markers is still a problem with targeting CSCs. However, AuNPs may also be used in photo-thermal therapy (PTT) due to their plasmon resonance and the tumor's retention properties. Here, AuNPs accumulate over time in the tumor after which excitation with light induces hyperthermia. This approach could hold great promise in reversing treatment resistance with minimal to no off-target effects. Here, we discuss CSC properties, surface markers and novel techniques and how they may aid in reversing therapy resistance.

Introduction

According to data published by the World Health Organization (WHO), cancer is the second leading cause of death worldwide. In 2018 alone, an approximate 9.6 million people died from this disease. Cancer may emerge in virtually all types of tissues and has the capability to metastasize throughout the body, making it harder to eradicate malignant cells. [1] Furthermore, intricate genetic, epigenetic and metabolic differences between cancer subpopulations lead to heterogenous neoplasms, meaning that different cancer phenotypes are present within one tumor. Therefore, making it more complicated to find antitumorigenic therapies that are efficient in eliminating whole tumors since they are often specific and do not target all subpopulations.

There are two possible explanations for tumor heterogenicity. The first one is clonal evolution, where genetic and epigenetic mutations result in genetic diversification and thus diverse cancer subpopulations. The second possibility for cancer heterogenicity, which will be discussed in this literature review involves cancer stem cells (CSCs). [2] Normal stem cells have the capability to self-renewal and are able to differentiate into more specialized cell types. Similarly, to normal stem cells, CSCs also have the ability to self-renewal and differentiate into new distinct cancer cell types. Using asymmetric cell division, they are able to maintain the CSC population whilst also giving rise to newly differentiated cancer cell types. It is believed that CSCs are the root of the tumor. [3] There are two different theoretical models that explain the presence of CSCs. The stochastic model describes how every cancer cell through genetic mutations can become a CSC, meaning that they have an equal probability of initiating tumor formation and unconstrained proliferation. Most current treatments are based on this model. However, some patients - receiving the same treatment – relapse, while others do not. The hierarchical model, also known as the cancer stem cell model, may explain as to why this is. It postulates that there is a single subpopulation of CSCs that gives rise to all different types of cancer cells and that only this CSC population holds the capacity to metastasize and initiate growth of new tumors. The majority of these tumors consist of diversely differentiated cancer cells derived from this CSC population and primarily contribute to tumor growth, whilst CSCs are associated with restricted proliferation. It should be noted that both models may hold truth and neither need to be wrong. [4]

Researchers have found that CSCs are often resistant to anti-cancer therapies. There are a number of explanations as to why this is. For instance, as mentioned in the previous paragraph, CSCs are often in a quiescent state although this may differ between cancers. However, most conventional chemotherapeutic agents target cells with a high proliferation rate, therefore not affecting slow proliferating CSCs. Their drug-efflux ability using ABC transporters also makes them less sensitive to anti-cancer drugs. Furthermore, overexpression of DNA-repair mechanisms in combination with inhibition of apoptosis pathways makes CSCs less susceptible to stress. Lastly, some types of CSCs, often found in the periphery of the tumor, have an epithelial-to-mesenchymal transition (EMT) phenotype which is associated with invasive and metastatic properties. [2] EMT normally occurs during the embryonic developmental stage and transforms epithelial cells into mesenchymal cells and inhibiting this process has shown to cause serious developmental defects. [5] Mesenchymal cells are highly motile and invasive. As one can conclude, these characteristics in malignant cells promotes metastasis. [2]

For these reasons, most conventional radio- and chemotherapies are insufficient in exterminating all malignant cells. Though initially they might show promising results, only very few CSCs have to survive in order for tumor relapse to occur as depicted in *figure 1*. Hence,

new strategies are under development to either eliminate CSCs or to inhibit stemness and metabolic pathways, thus reversing therapy resistance. These approaches often focus on identification of specific CSC surface markers and stemness pathways. Targeting stemness and self-renewal pathways to push CSCs into a differentiated cancer cell state could make them more susceptible to conventional therapies. Hence, the goal of this review is to explore what novel anti-cancer treatments hold promise in targeting CSCs. In order to reach this goal, a look is taken at the different stemness pathways responsible for resistance and what advancements are made in targeting these regulatory systems. Another strategy, used for targeting CSCs and drug delivery, is the use of CSC surface markers. Some of the most extensively research surface markers will be addressed in this review. Subsequently, a look is taken at how these surface markers may be used in drug delivery and how they might be utilized in gold nanoparticle treatments, after which the advantages will be mapped out to the current problems with these new strategies. Lastly, the use of RNA interference to target stemness pathways in tumor cells will be discussed.

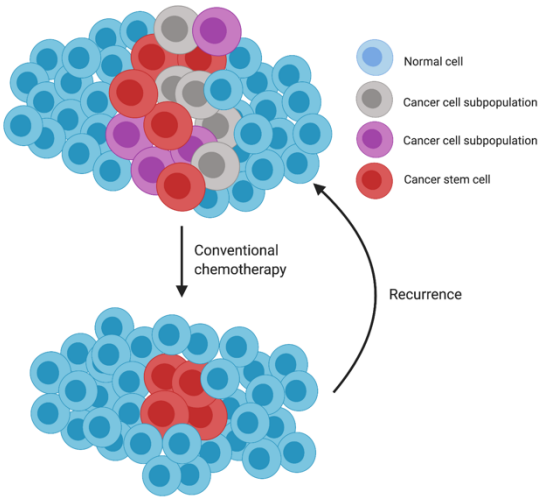

Figure 1. The cancer's response to conventional chemotherapeutic treatments initially appears to effectively diminish the bulk tumor. However, therapeutic resistance of CSCs induces relapse.

Stemness pathways responsible for therapy resistance

Stemness can be defined as the ability of a cell to differentiate into other cell types, thus changing its phenotype and most importantly, self-renewal. Cells exhibiting stemness are capable of interacting with the microenvironment to preserve a delicate balance between proliferation, regeneration and quiescence. Normal stem cells are unable to survive outside of their microenvironment due to a lack of specific factors and cytokines. Through asymmetric cell division, they play a crucial role in tissue regeneration and stability whilst also maintaining the stem cell population. This type of cell division delivers tissue specific progenitor cells that create daughter cells responsible for replacing damaged tissue. CSCs exhibit many similar stemness features compared to healthy SCs making them malignant equivalents. Shared features are a high capacity of self-renewal, slow cell-cycles, cellular plasticity and the ability to differentiate and dedifferentiate. In addition to these intrinsic features, both cell types thrive in a protective microenvironment. Due to these stem cell-like characteristics, CSCs are creating a heterogenous malignant tumor. Furthermore, stemness features of CSCs make them more resilient to stresses. The tumor microenvironment promotes persistence of cancers through selection due to the hostile circumstances produced by chemotherapeutic drugs, radiation stress, hypoxia and the immune system. As a consequence, malignant cells surviving these stresses become even more resistant. [6] Hence, researchers have been focusing on targeting stemness pathways in order to make them susceptible to anti-cancer treatments or eliminate them all together. Some of these recent advancements are described below.

Current advancements in targeting stemness pathways to reduce therapy resistance

An important pathway involved in stemness is the phosphatidylinositol-3-kinase (PI3K) signaling transduction pathway. It is responsible for self-renewal, proliferation, cell survival and growth. A key inhibitor of this pathway is Phosphatase and tensin homolog deleted on chromosome ten (PTEN), which hydrolyzes phosphatidylinositol (3,4,5)-trisphosphate (PIP3) into phosphatidylinositol 4,5 phosphate (PIP2) thereby blocking the activation of downstream effector molecules in the PI3K pathway. A lack of PTEN expression has frequently been observed in cancer patients due to mutations, deletions or transcriptional silencing. Therefore, inducing PTEN expression in malignancies lacking sufficient expression levels could hold potential in antagonizing the PI3K signaling pathway and reducing stemness in CSCs. [7] For example, in Qi et al., the tumor suppressive characteristics of PTEN were demonstrated in *PTEN* Knock-down (KD) and transfected human breast cancer cells. A lack of this lipid phosphatase was associated with an increased expression of a PTEN substrate called Abelson interactor 1 (Abi1), which induced EMT and CSC activity. Abi1 is known to be an adaptor protein for stabilization of the WAVE regulatory complex, playing a crucial role in actin nucleation and formation of the cytoskeleton. Overexpression of Abi1 *in vitro* lead to a decrease in epithelial cadherin (E-cadherin) and an increase in transcription factors associated with EMT. Furthermore, overexpression or even transient expression of PTEN induced mesenchymal to epithelial transition (MET) in these breast cancer cells. This data indicates that PTEN plays an important role in decreasing invasiveness through EMT reversal of CSCs. [8]

The JAK/STAT pathway has also been associated with CSCs, playing an important role in self-renewal. [2] In a study by Dolatabadi et al., the canonical JAK/STAT signaling pathway was investigated in the context of myxoid liposarcoma (MLS) CSCs. This study showed that a Leukemia inhibitory factor (LIF) induced the formation of CSCs through the JAK/STAT pathway, whereas inhibition of this pathway through blocking phosphorylation of STAT3 with ruxolitinib - inhibits JAK1/2, which is responsible for STAT3 phosphorylation - showed increased susceptibility to the chemotherapeutic compound doxorubicin. Emphasizing the importance of the JAK/STAT pathway for chemoresistance. [9]

NFκβ pathway, in addition to the PI3K and JAK/STAT pathways, is also involved in proliferation, migration and activation of anti-apoptotic pathways. Increased NFκβ activity in breast CSCs has been associated with stemness and invasiveness. [2] Zakaria et al. reported that NFκβ inhibition using a compound called BMS-345541 reduced stemness and EMT of Lung CSCs through binding the allosteric site of the transcription factor, thereby blocking NFκβ-mediated transcription. However, therapeutic efficacy and specificity of this inhibitory drug on patients has yet to be assessed. [10]

A crucial pathway that is more preferentially activated in CSCs is the Sonic Hedgehog (SHH) signaling pathway, which is linked to an EMT phenotype, CSC survival and progression. [11] This stemness pathway, when inhibited, is associated with tumor regression. In Li et al., a drug extracted from soybeans called genistein was used to inhibit the SHH signaling pathway in renal CSCs. The data showed that this drug not only inhibited the SHH signaling pathway and its target genes, but also lead to a decrease of CSC markers in tumor spheres and an increase in apoptotic markers, suggesting that genistein has anti-tumorigenic potency. Genistein-mediated SHH inhibition is known to be accomplished through downregulating Shh, Smoothened, frizzled class receptor (SMO) and Glioma-associated oncogene transcription factor family (GLI) GLI1/2. However, the exact molecular mechanisms behind genistein-mediated inhibition have yet to be elucidated. [12] More recently, Yang et al. reported the

importance of SHH in CSC persistence by utilizing a microRNA called miR-135a and siRNAs mimicking miR-135a function. They were designed to target SMO, which is a G-protein coupled receptor and crucial for SHH signal transduction by activating GLI proteins which in term leads targets gene transcription. Overexpression of miR-135a, thus silencing of SMO, lead to a decrease in cell viability and an increase in apoptosis compared to the non-coding siRNA and blank groups. [13]

Another important regulatory pathway called the Wnt/β-catenin signaling pathway has been reported to be preferentially expressed in CSCs. When Wnt signaling is activated, β-catenin migrates into the nucleus and initiates transcription of a whole range of downstream targets responsible for proliferation, differentiation, and migration. Aberrant regulation of β-catenin leads to insufficient ubiquitination and overexpression of target genes. A number of different inhibitors that target different parts of the pathways have been identified. One such inhibitor targeting the interactions between β-catenin and TCF4 transcription factor, called LF3 which is a 4-thioureido-benzenesulfonamide derivative, has shown to reduce tumor growth and self-renewal capabilities and induce differentiation of colon cancer in a mouse xenograft model. [14]

Lastly, the NOTCH signaling pathway, primarily expressed in stem cells and important for tissue development and homeostasis is often expressed in different types of CSCs. [15] This pathway has been associated with CSC progression, tumor immunity, angiogenesis and metastasis. The NOTCH signaling pathway consists of four receptors and five different ligands. Once the receptor binds a ligand, the notch intracellular domain is cleaved by γ-secretase inside the nucleus after which transcription of NOTCH target genes takes place. Most inhibitors that are currently used in targeting the NOTCH pathway focus on γ-secretase or NOTCH ligands. PF-03084014, is a drug that targets γ-secretase function and consequently antagonizes NOTCH signaling. It has shown to inhibit self-renewal and proliferation whilst inducing differentiation in hepatocellular carcinomas. [15,16]

Advancements in clinical translation of Wnt and NOTCH signaling pathways

Even though there are currently very few therapies on the market for targeting CSCs, some promising treatments are in trial that may hold merit.

Zhang et al. lined out a whole range of pre-clinical and clinical trials – some even in phase 2 - on a number of different agents used to inhibit certain parts of the Wnt/β-catenin pathway. Some of these agents show promising results although further data needs to be collected on efficacy and off-target toxicity. [13] For the NOTCH signaling pathway there are a number of inhibitors in phase 1 or 2 clinical trials as well, one of these drugs as previously mentioned PF-03084014 is in phase 2. This drug in combination with gemcitabine and nab-paclitaxel, conventional chemotherapeutic drugs, have shown to increase the life expectancy of patients with metastatic adenocarcinomas. According to Yang et al., these drugs could potentially aid in reversing therapy resistance and, especially in combination, with conventional chemotherapy help in preventing relapse. [16]

Considering the preclinical and clinical data that is out there, these stemness pathways appear to be potential targets in reducing stemness and reversing therapy resistance. However, a problem with targeting these signaling pathways is that they are often expressed ins heathy cells as well. Therefore, it is important to assess toxicity and unsought side effects. Pathways that have shown to be more specific for CSCs are primarily Wnt/β-catenin, SHH and Notch with less side effect. Making them more suitable candidates for further research and clinical trials. [2,16]

Post-transcriptional regulatory pathways targeting stemness

RNA interference (RNAi) is a technique in which synthetic small non-coding RNA molecules or short-interference RNAs (siRNA) are used to regulate gene expression. As discussed previously, CSCs often exhibit aberrant gene expression which leads to dysregulated function of certain molecular pathways. miRNAs are endogenous non-coding oligonucleotides of approximately 22 base pairs that play an important role in regulating gene expression post-transcriptionally. They play a vital role in all aspects of cell development and are known to be crucial for stem cell function, proliferation, differentiation, cell cycle control and metabolism. Aberrant expression of these miRNAs that are associated with CSCs and stemness pathways may therefore be a potential target in reversing self-renewal, tumorgenicity and chemoresistance. This can be achieved by either inducing transcription of the miRNAs, or potentially generating siRNAs mimicking the miRNA function. [17]

There is already extensive evidence for the role of miRNAs in a number of different types of cancers. For instance, miR-34a has been shown to be tumor suppressive in brain tumors and glioma stem cells and is downregulated in glioma tumors. It inhibits cell cycle progression, proliferation and invasion by targeting the NOTCH signaling pathway. [18] Furthermore, miR-34a is associated with controlling symmetric and asymmetric division in colorectal CSCs. Increased miR-34a expression antagonized NOTCH signaling and led to non-CSC progenies. [19] In Garzia et al., the NOTCH signaling pathway was targeted in medulloblastoma using miR-199-5p leading to depletion of $CD133^+$ CSCs. [20] Moreover, in breast CSCs, overexpression of miR-7 has been reported to decrease CD44 expression and inhibits the NFκβ pathways, and as a consequence inhibits growth of breast CSCs. [21] Emphasizing again how expression of miRNAs can affect the CSC population, and these findings support the idea that future therapies could be based in regulating its expression. Therefore, a promising approach would be to deliver siRNAs mimicking specific miRNA's function or miRNA inhibitors to tumors in order to eliminate CSCs by antagonizing its stemness pathways. This would make them more susceptible to anti-cancer drugs. Another reason why CSCs are chemo resistant is their ability to transport chemotherapeutic drugs out of the cell via drug efflux pumps like ABC transporters, which are known to be activated in CSCs. [17] For example, a decrease in the expression of miR-451 in colon cancer is associate with an increased expression of ABCB1 membrane transporter making these cancer cells less sensitive to a chemotherapeutic drug called irinotecan. Interestingly, after restoring miR-451 expression, cells were susceptible for this drug again. In addition, data showed that colon cancer cells with restored miR-451 expression exhibited a decrease in self-renewal potential and cell proliferation. [22]
These finding suggest that miRNA-based therapies, that is, targeting stemness pathways through altering miRNA expression patterns, may be a powerful tool in targeting CSCs. However, some problems with this approach need to be further investigated. The primary obstacle is the delivery of siRNAs *in vivo* with high specificity and limited off-target effects. Therefore, no commercial therapies are currently available on the market. A possible solution could be to conjugate small oligonucleotides to nanoparticles, which will be discussed in a later paragraph.

Identification of CSC surface markers for therapeutic targeting

In addition to specific stemness regulatory pathway, cell surface markers specific for CSCs may be used for therapeutic targeting. These surface markers are often proteins or carbohydrates attached to the cell membrane. They serve a specific function such as signaling or transportation of molecules. In addition to these biochemical functions, some surface markers are specific for a particular cell type, making them suitable candidates for identification. After identification, CSC specific markers may be used as candidates to target and interrupt certain cell processes. However, different cancer cell types have their own specific markers and heterogeneity of tumors means that this strategy is often cancer specific. Furthermore, these surface markers are rarely only seen in CSCs and are in many cases also expressed in healthy tissue, raising the possibility of off-target effects when designing treatments based on this approach. Some of the most extensively studied CSC markers are listed in table 1 and will be discussed in this paragraph.

Specific CSC markers

Cluster of differentiation antigens or CD molecules are one of the most common types of antigens studied. They play an important role in immune communication as either receptors or antigens. [23] In Heng et al., CD44 was described as a potential marker for small cell lung carcinoma, claiming that increased expression was perceived in CSCs in later stages of progression. CD44 was often colocalized with two important CSC but also SC markers; MYC and SOX2, again emphasizing CD44's potential to serve as a CSC marker. [24] This surface marker is a hyaluronic acid receptor involved in cell adhesion, cell-cell interaction, migration, drug resistance and apoptosis. [2,25] A study in Zhang et al. found a highly tumorigenic subpopulation in human ovarian serous adenocarcinomas double positive for CD44 and CD117 were found to form and identical tumor upon transplantation into mice, whereas $CD44^-/CD117^-$ cells after transplantation were not able to form tumors. [26] In another study on ovarian cancer CD133 was reported to be overexpressed in tumor initiating cells with increased chemotherapeutic resistance properties giving rise to $CD133^-$ cells. [27] CD117 and CD133 are stem cell growth factor receptors and hematopoietic stem cell markers [2,27] and together with CD44 are described as CSC markers resistant to conventional chemotherapeutic drugs. [2,27,28] Knocking down CD44 in ovarian cancer has shown to reverse drug resistance and decrease tumorgenicity [29], however a preclinical attempt to knock down CD117 through inhibition of the Wnt/β-catenin pathway using imatinib had no beneficial results possibly due to a lack of specificity within the tumor. [30] CD133 positive cells have been reported to be present in a number of different types of tumors including breast, prostate, liver, colon, pancreatic, lung and head and neck squamous cell carcinomas. Another approach could be drug delivery using antibodies to target these surface markers.

Toxin-conjugated antibodies

Drug delivery specifically targeting $CD133^+$ cells using Anti-$CD133^+$ Single-chain variable fragments (scFv) has been proposed in Waldron et al. A deimmunized targeted toxin called CD133KDEL was used to target the extracellular domain of CD133, with KDEL standing for the amino acid chain that was added to the C-terminal for enhanced drug activity in the endoplasmatic reticulum (ER). This toxin was synthesized using an anti-CD133 scFv containing a PE38 toxin and was able to bind all six isoforms. Human head and neck cancer was treated with dCD133KDEL *in vitro* and *in vivo*. Intratumoral injections lead to growth inhibition and complete remission. [30,31] Another approach which has shown to be even more effective, is constructing a dual targeting toxin. This has a few different advantages. First of all, cancer cells often exhibit immune escape mechanisms with which malignant cells discard antigens. Furthermore, tumors, as discussed previously, are heterogeneous with different subpopulation, which complicates targeting. Lastly, targeting multiple binding sites may increase affinity of the toxin-conjugated antibody. This concept was put to the test in Waldron et al. (2013), with a single chain targeted toxin called EpCAMCD133KDEL consisting of an anti-CD133 scFv linked to an anti-EpCAM scFv again with the PE38 toxin. Epithelial cell adhesion molecule (EpCAM) is another CSC marker which plays an important role in proliferation, differentiation, migration and cell signaling. Over expression of this marker has been associated with increased tumorigenesis. This bispecific construct caused complete remission in head and neck cancer *in vivo* model and inhibited translation and proliferation *in vitro*. [30,32] More recently, in Yan et al. a prostate marker γ-seminoprotein (γ-SM) was used in a dual-targeting construct. Here, mesenchymal stem cells (MSCs) were engineered to produce a pro-apoptotic dual-targeting construct consisting of an anti-γ-SM scFv with a short furin cleavage sequence form diphtheria toxin and an activated pro-apoptotic truncated bid (tBid) referred to as MSC.scFv-Fdt-tBid. These cells migrated effectively to the tumor sites and continually released the scFv-Fdt-tBid construct. Accumulation of this construct at the tumor site was observed with anti-tumorigenic effects, again elucidating the effectiveness of dual-targeting constructs.
However, this approach has its downsides since most tumors have poor perfusion and this technique must be administered intravenously. [33] Hypoperfusion therefore results in limited drug delivery. A solution to this problem might be to combine this therapy with conventional chemotherapy. The bulk of the tumor would be eliminated by chemotherapeutic drugs thus making it easier for the toxin-conjugated antibodies to reach the CSCs. However, this has not yet been investigated in a clinical setting. [30] Currently, there are a number of toxin-conjugated antibodies in clinical trial. A clinical trial that ended in 2021 concerning a toxin-conjugated antibody targeting Folate receptor alpha positive tumors showed anti-tumorigenic effects with low toxic side-effects in patients. Exemplifying the promising possibilities of toxin-conjugated therapies. [34]

Table 1. Overview of specific CSC markers

CSC marker	Phenotype	Cancer	Reference
CD44	Tumorigenicity, spheroid formation, chemoresistance, hierarchical organization, proliferation	Ovary, stomach, breast, liver, head and neck, colon, prostate, pancreas, SCLC	[2] [23] [27]
CD117	Tumorigenicity, spheroid formation, self-renewal, chemoresistance, hierarchical organization, undifferentiated state	ovary, skin, colon, blood, head and neck, sarcoma, germ cells tumors, prostate, lung, mesothelioma, breast, renal,	[2] [27]
CD133	Poorly differentiated gastric cancer, independent prognostic factor	Blood, ovary, brain, pancreas, liver, skin, prostate, colon, lung, stomach, head and neck	[2] [26] [31]
EpCAM	Tumorigenicity, phenotypical heterogeneity, self-renewal, metastasis, chemoresistance	Stomach, ovary, pancreas	[2] [32]

Taken together, CSC surface markers hold potential to be used for anti-cancer treatment. However, a lack of specific surface markers makes it complicated to precisely eliminate a certain cell population without off-target cytotoxicity. CD44 and CD133 have both been described as CSC surface markers but are also known to be expressed by healthy cells. These problems may be avoided by designing bispecific antibody-conjugated toxins in combination with chemotherapeutic drugs.

Targeting CSCs using gold nanoparticles

To overcome the above-mentioned drawbacks associated with targeting CSCs based on their surface markers might be the use of gold nanoparticles. Proper delivery of anti-tumorigenic drugs to CSCs has shown to be a problem. Furthermore, CSCs are often resistant to drugs and consequently, higher concentrations of chemotherapeutic agents are required to kill them leading to cytotoxicity of healthy tissue. Active drug delivery, however, is a strategy in which specific cells are targeted with drugs using nanoparticles. This approach addresses the problem of delivery and cytotoxic side effect. There are several different types of nanoparticles used in biomedical science, but a new type called gold nanoparticles (AuNPs) hold great promise for future anti-cancer treatment. [2]

Drug delivery using AuNPs can both be active or passive. Active drug delivery is accomplished by using membrane markers and ligands that are recognized by antibodies or aptamers whereas passive drug deliver makes use of enhanced retention and permeability properties of tumors. AuNPs used in biomedical science are conjugated to functional groups such as antibodies, peptides, aptamers, fluorescent dies, polyethylene glycol (PEG) or cytotoxic drugs. PEG is used to increase the particle's half-life by decreasing uptake by phagocytic cells that reside in the tissue. [2,35] Gold has a whole range of different physical and chemical properties making it a suitable compound for biomedical applications. Recent developments have made it possible to easily couple AuNPs to Sulfur and Nitrogen-containing organic molecules making them biocompatible and together with increased retention of tumors increases target specificity. Another property that is physical in nature is gold's surface plasmon resonance, which enables it to convert near infra-red light into heat. [36] This type of light with a wavelength between 450 and 600 nm can penetrate deep into the body with minimal absorption. Cancer cells containing accumulated AuNPs then absorb the light and convert it into heat, after which the affected cells undergo hyperthermia and die. This method is called photo-thermal therapy (PTT), which could be an effective way in killing both CSCs and cancer cells in general. Furthermore, this strategy is highly specific with limited effect on healthy surrounding tissue mitigating cytotoxicity since the surrounding tissue does not contain accumulated AuNPs. Considering AuNPs versatility, combining these different techniques (i.e., antibodies, toxins and PTT) may increase tumor cytotoxicity. *Figure 2* shows the schematics of how antibody conjugated AuNPs may be used for targeting tumor resistant tumors using PTT in combination with chemotherapy.

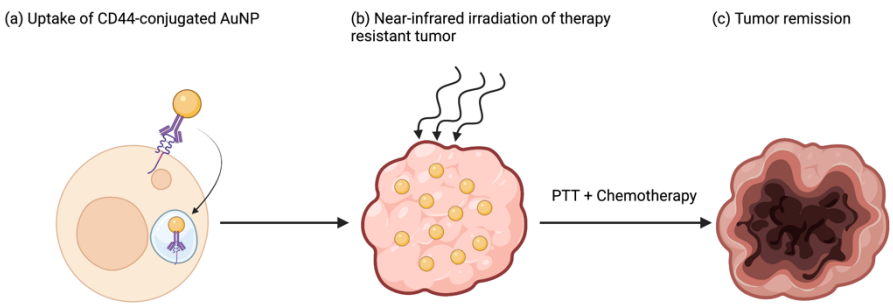

Figure 2. The use of CSC markers, PTT, and chemotherapy to target therapy resistant tumors.
(a) Uptake of anti-CD44-conjugated AuNP by a $CD44^+$ breast cancer cell. (b) PTT on therapy resistant tumor with accumulated AuNPs. (c) Combining PTT with conventional chemotherapeutic drugs leads to complete remission.

PEGylated AuNPs containing a CD44 antibody has shown to be successful in targeting human breast CSCs in vitro. In Patskovsky et al., the 3D localization of CSCs was mapped with AuNPs *in vitro* using hyperspectral darkfield microscopy. The data showed successful uptake of CD44-AuNPs by CD44-expressing breast CSCs. [37] Another study, published by Liang et al. used CD44 isoform v6 conjugated gold nanoparticles for photoacoustic imaging and PTT in gastric CSCs. [38] Photoacoustic imaging is a technique in which light energy absorbed by tissue results in expansion due to light-to-heat conversion. This expansion creates ultrasound waves which can be used to generate images using ultrasonic transducers. [39] This study showed successful uptake of CD44v6-AuNPs by gastric CSCs and complete elimination *in vitro* after exposure to near infra-red light, indicating again the advantage of combining the use of antibodies targeting CSC markers and PTT. Furthermore, photoacoustic imaging using these AuNPs injected intravenously in nude mice, showed successful targeting of the gastric cancer vascular system. Subsequently, exposure to near infra-red light led to growth inhibition and elongated lifespans of the treated mice. [38] In a similar study, breast cancer cells exhibiting increased expression of a growth hormone called HER2 were targeted using anit-HER2-AuNP. This type of cancer often displays resistance for an anti-HER2 drug called trastuzumab. The data showed sufficient accumulation of these particles in 72 hours and PTT ensued growth inhibition and tumor regression. Emphasizing again that PTT is a successful candidate in reversing drug resistance [40] In another study, glioblastoma CSCs were successfully targeted using AuNPs conjugated to a small peptide recognizing CD133. Small peptides are less immunogenic and toxic with a similar binding affinity compared to antibodies. In addition, small peptides penetrate cells more easily due to their smaller size. [41] All things considered, AuNPs are enormously versatile and a suitable candidate for anti-cancer therapies. PTT, especially in combination with active targeting using antibodies, has shown to be effective in eliminating CSCs where conventional chemotherapeutic agents cannot. However, as of yet, very few studies have been conducted regarding AuNP drug delivery. Furthermore, no Gold-based nanoparticle therapies have be approved by the Food and Drug Administration (FDA). Therefore, it can be said that gold-based nanomedicine is still in its infancy, but with promising translational potential.

Conclusion & future perspectives

Chemoresistance in CSCs has shown to be a serious obstacle in treating patients with cancer. Tumor relapse may occur when not all CSCs are irradicated, even after seemingly effective treatment of cancers. Therefore, it is of the essence to target this subpopulation. In this review a look is taken at the molecular pathways responsible for the CSC phenotype and some of the most promising novel strategies to target this cancer population.

In order to target CSCs, it is important to understand which signaling pathways are involved in maintaining stemness. There are a number of pathways reported to play a vital role in maintaining stemness in CSCs and targeting these pathways could help in reversing therapy resistance. And there are some things to take into consideration. For example, although aberrant function of the PI3K and JAK/STAT pathways are known to support CSC properties, targeting them may cause unwanted side effect. This is due to the fact that these pathways are also frequently expressed in healthy cells. [2,7] On the other hand, the Wnt/β-catenein, SHH and NOTCH signaling pathways have been shown to be more specific for CSC. [2] Therefore, treatments need to be highly specific and side effect always have to be taken into account. Determining suitable surface markers, in addition to the identification of stemness pathways, is another way in which CSCs can be identified. Extensive research has established a number of different potential CSC markers that could help in identifying the CSC population in cancers. However, there is a lack of specific surface markers since many of them are also expressed on non-CSCs. [2] A way to overcome this problem may be to use double marker targeting to rule out any off-target effect as is seen in Waldron et al. [31] Another approach in anti-cancer therapies that holds great potential for future treatment is the use of AuNPs. They can be used for imaging, drug delivery and PTT and, especially in combination of antibodies, may in term be an effective technique to specifically target CSCs and resolving therapy resistance. Although some problems concerning toxicity, and clearance of nanoparticles have yet to be addressed. It is highly likely that AuNPs will be used in the future treating cancer, however, the current progress is not anywhere near an actual clinically developed treatment. Lastly, growing evidence suggests that miRNAs play a crucial role in maintaining CSC properties and promising data has shown that promoting or inhibiting expression of these oligonucleotides leads to regression of cancer and CSCs. A next step would be to develop in vivo studies that address problems including administering miRNAs *in vivo* and any potential off-target effects it has.

All things considered, there is currently no 'Silver bullet' that is able to effectively target CSCs in patients without any negative side effects. A whole range of different approaches are currently being investigated and some therapies hold great promise in decreasing tumorgenicity, invasiveness and therapy resistance. Most of these techniques alone will probably not be enough and especially combination studies have shown to be effective. Increasing the specificity with which CSCs are identified and localized, together with PTT and conventional chemotherapeutic drugs could in term aid in minimizing the occurrence of relapse.

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