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Original Article

The use of natural products to target cancer stem cells

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Abstract: The cancer stem cell hypothesis has been used to explain many cancer complications resulting in poor patient outcomes including induced drug resistance, metastases to distant organs, and tumor recurrence. While the validity of the cancer stem cell model continues to be the cause of much scientific debate, a number of putative cancer stem cell markers have been identified making studies concerning the targeting of cancer stem cells possible. In this review, a number of identifying properties of cancer stem cells have been outlined including properties contributing to the drug resistance and metastatic potential commonly observed in supposed cancer stem cells. Due to cancer stem cells' numerous survival mechanisms, the diversity of cancer stem cell markers between cancer types and tissues, and the prevalence of cancer stem cell markers among healthy stem and somatic cells, it is likely that currently utilized treatments will continue to fail to eradicate cancer stem cells. The successful treatment of cancer stem cells will rely upon the development of anti-neoplastic drugs capable of influencing many cellular mechanisms simultaneously in order to prevent the survival of this evasive subpopulation. Natural compounds represent a historically rich source of novel, biologically active compounds which are able to interact with a large number of cellular targets while limiting the painful side-effects commonly associated with cancer treatment. A brief review of select natural products that have been demonstrated to diminish the clinically devastating properties of cancer stem cells or to induce cancer stem cell death is also presented.

Keywords: Cancer stem cells, drug resistance, metastasis, natural products, drug discovery, cancer recurrence, polyphenols, alkaloids, flavonoids, chemotherapy

Introduction

Modern chemotherapy, radiotherapy, and other antineoplastic regimens have made the treatment of many solid tumors possible and have given hope to those diagnosed with cancer. However, the prognosis for many cancer patients remains bleak due to the high rate of cancer recurrence and multiple drug resistance (MDR) seen after initial chemotherapy treatments. Metastatic cancers affecting multiple organ systems are particularly difficult to treat and oftentimes demand the partial or complete surgical resection of multiple tissues. Cancer stem cells (CSCs) potentially explain many of the shortcomings of established chemotherapy treatments.

CSCs are distinguished as a small population of tumor cells which are able to form phenotypically diverse tumors, as wells as self-renew and differentiate. They are described as belonging to a group of tumor initiating cells (TICs) which may or may not possess stem-like characteristics, but debate remains as to how large a proportion of TICs are indeed stem-like. Additionally, it is not clear whether or not the plasticity of tumor cells allows any cell to become stem-like and gain the capability to recapitulate heterogeneous tumors. The role of CSCs in tumor formation was first identified by Bonnet and Dick in the late 90s [1]. In this paper, the $CD34^+/CD38^-$ subpopulation of cells from acute myeloid leukemia were shown to form tumors in immunodeficient NOD/SCID mice with higher efficiency than the $CD34^+/CD38^+$ subpopulation. The ability of CSCs to asymmetrically divide, allowing the CSC to self-renew as well as differentiate to produce a heterogeneous tumor containing multiple cell phenotypes, was also identified. Since this discovery, the CSC hypothesis has been tested rigorously, and evidence that CSCs

play a crucial role in tumor development for many different cancers has been reported. These include breast carcinoma [2, 3], colorectal carcinoma [4], head and neck squamous cell carcinoma [5], hepatocellular carcinoma [6, 7], lung carcinoma [8], ovarian adenocarcinoma [9], glioblastoma [10], and pancreatic carcinoma [11] among others.

According to the CSC model, cancer recurrence after treatment is due to the superior resistance of CSCs to cellular toxins and insults. While current treatments are capable of eradicating the bulk of the tumor mass, the lingering CSCs are able to form new, fully developed tumors from a small number of cells or even a single cell. CSCs are thought to resist treatment through several cellular mechanisms including the overexpression of drug efflux pumps, quiescence, and detoxifying enzymes [12]. A high population of CSCs within a tumor has subsequently been linked to MDR and a poorer prognosis for cancer patients [13]. Furthermore, the cellular machinery of CSCs has been shown to allow for epithelial-mesenchymal transition (EMT), a process by which epithelial cells lose their cell-to-cell and/or cell-to-matrix adhesion and can survive in a migratory state [14]. By undergoing EMT, migrating to other organs, and reattaching by mesenchymal-epithelial transition (MET), CSCs are hypothesized to initiate the formation of metastatic tumors.

Current methods for the treatment of cancer have been demonstrated to be insufficient in eliminating CSC populations from a number of cancer types. $CD133^+$ glioma CSCs have been shown to resist radiation therapy to a higher degree than their $CD133^-$ counterparts [15]. Breast CSCs exhibit a similar resistance to radiotherapy in addition to common chemotherapy treatments [16, 17]. Furthermore, the CSC population in residual breast cancer tumors has been shown to increase significantly following chemotherapy treatments, nearly doubling the tumorigenic potential of the residual cancer cells in immunodeficient SCID mice [17]. Treatments targeting a specific molecule or surface marker are likely to fail to eliminate CSCs due to the multiple survival pathways activated in CSCs in addition to the ambiguity of CSC markers across different tissue types, the presence of commonly used CSC markers in healthy tissues, and the often required combination of markers used to denote CSC populations. Treatments capable of reducing CSC populations will therefore require the development of novel, diverse, and multi-targeted approaches for cancer treatment. Due to the numerous, still poorly understood characteristics of CSCs, the discovery of CSC targeting therapies will likely be the result of opportunistic screening of new or known compounds against CSC populations.

Natural products may be the key to discovering novel treatments demanded by the difficulty of treating CSCs. Natural products (NPs) have been a historically rich source of biologically active compounds for the pharmaceutical industry. The value of NPs in medicine is a result of their ability to influence multiple signaling pathways simultaneously while producing diminished, benign side effects. The success of these compounds, especially as they relate to cancer treatment, has led researchers to investigate the effect of a number of NPs on CSCs. **Figure 1** summarizes the role of CSCs in cancer formation, metastasis, and relapse in addition to the potential role of natural products in their treatment. In this review, properties distinguishing CSCs as well as properties which give rise to the drug resistance associated with CSCs are identified. A brief review of select NPs which have been shown to target CSCs is also provided.

Identifying cancer stem cells

One of the major challenges facing cancer stem cell research is accurately defining which tumor cell subpopulations are stem like. The gold standard for identifying CSCs remains the ability of a small number of cells to generate a fully developed tumor when injected into immunocompromised mice, but the cost, time, and labor associated with animal studies have led to the search for markers of stem like cancer cells. Many putative CSC markers have been proposed and subsequently identified as targets for chemotherapeutics. However, the expression of these markers has been shown to be inconsistent across CSCs from different tissues and tumor phenotypes [18, 19]. Additionally, many of the reported CSC markers are possessed by healthy stem cells and even non-cancerous, non-stem-like cells, posing a challenge to the development of targeted therapies based upon these markers.

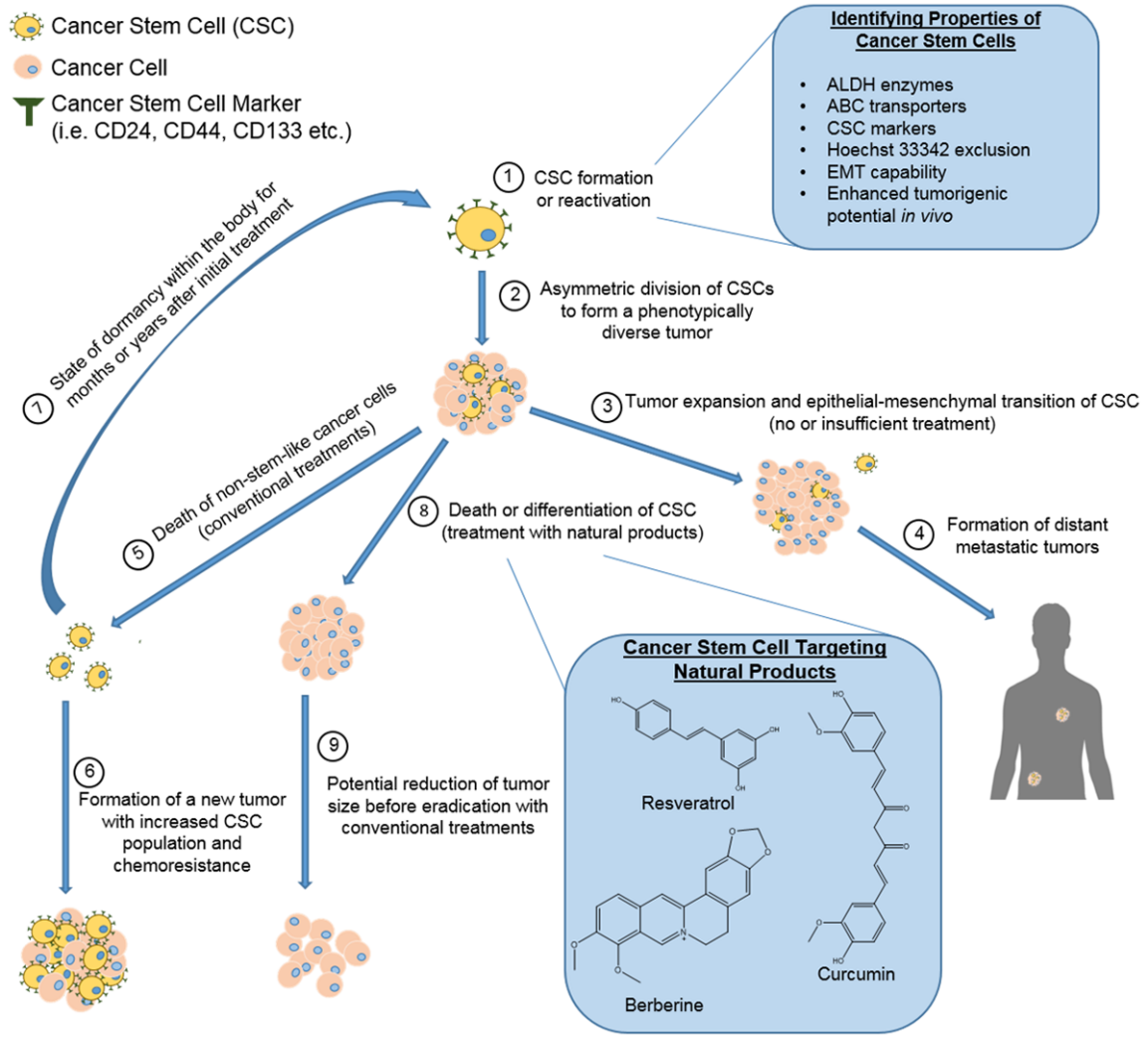

Figure 1. Illustration of the Cancer Stem Cell Model's explanation for tumor formation, metastasis, and recurrence and the potential of natural products in their treatment. Cancer Stem Cells (CSCs) are either formed upon carcinogenesis of somatic cells or stem cells, or they are activated after a period of dormancy (1). These CSCs then asymmetrically divide resulting in a phenotypically diverse tumor consisting of both CSCs and non-stem-like cells (2). Left untreated, the tumor will continue to grow and invade the surrounding tissue, and CSCs undergoing EMT may break off from the original tumor and travel to distant organs (3). The CSCs which reattach throughout the body can then initiate a new tumor, resulting in metastases (4). Using current treatment methods capable of inducing cell death in the bulk of tumor cells, the CSCs are not destroyed due to their enhanced survival traits, such as quiescence and the expression of ALDH enzymes and ABC transporters (5). The remaining CSCs may then go on to recreate the original tumor, sometimes increasing the percentage of CSCs within the tumor and forming multiple drug resistant tumors (6). In other cases, the remaining CSCs will enter a state of dormancy within the body and remain undetected for long periods of time before reactivating and initiating the formation of a new tumor, thus resulting in cancer relapse in patients thought to be cancer free (7). As a result of these issues, new treatments are being investigated which can target CSCs. Natural products have shown the potential to induce cell death in CSCs, cause CSCs to differentiate, or sensitize CSCs to conventional chemotherapy treatments (8). Once the CSCs have been eliminated, the remaining tumor may diminish in size and can be subsequently eradicated through the use of conventional antineoplastic therapies (9).

Oftentimes, a combination of supposed CSC markers is required to denote the CSC population. For example, a common population of cells within breast cancer that has been deemed breast cancer stem cells are $CD44^+/CD24^-/ESA^+$ [2]. The most notable among these putative markers are the surface proteins CD44 and CD133 which have been used to identify CSCs in a wide array of cancer types. In addition to these supposed markers, certain

properties of CSCs have also been use to distinguish them from the rest of the tumor population. For example, cells known as the side population (SP) have been shown to possess a high percentage of TICs [7, 9, 10]. Tumors cells within the SP are distinguished by their ability to exclude Hoechst 33342 fluorescent stain which is typically assessed via flow cytometry.

Investigators have attempted to isolate populations of CSCs using these properties combined with flow cytometric techniques or selective growth environments. Using these purified populations of CSCs, their tumorigenic properties and specific responses to drug candidates can be better investigated. Low purity of isolated populations, the ability of CSCs to differentiate into phenotypically diverse populations, and disagreement over which markers should be used to identify CSCs still pose major hurdles to many of these techniques. A brief review of common CSC markers and characteristics used to identify or isolate CSC populations is provided below.

CD44

Cluster of differentiation 44 (CD44) is a very commonly utilized marker for CSCs. CD44 proteins are integral membrane glycoproteins which play a role in cell attachment to the extracellular matrix by binding to hyaluronan (HA). CD44 is often used in combination with other markers to denote CSCs; however, in cases such as head and neck squamous cell carcinoma, CD44 has also been used alone to identify cancer cells capable of self-renewal and differentiation [5]. The expression of this marker has been used as a putative marker for cancer stem cells in such tissues as breast [2], ovarian [20], pancreatic [21], and bladder [22] along with many others. CD44 regulates the growth, migration, and invasion characteristic of CSCs in addition to modifying the extracellular matrix of tissues to support new tumor formation [23, 24]. Interestingly, cells expressing CD44 also produce a higher amount of the cytokine transforming growth factor beta (TGF-ß) which has been shown to aid EMT [25]. Further, HA-CD44 binding activates protein kinase C_e, which in turn phosphorylates the stem cell maintenance transcription factor, Nanog. Nanog then begins a signaling cascade which results in the upregulation of ATP binding cassette B1 (ABCB1), a drug efflux pump, contributing to MDR [24]. Reducing the population of CD44 expressing cells in tumor populations, therefore, has the potential to diminish the CSC population and limit invasion, metastases, and drug resistance in a broad spectrum of cancers.

CD133

Cluster of differentiation 133 (CD133) is a pentaspan surface membrane protein that is also commonly used as an indicator of CSCs. Interest in this marker as an indicator of CSC was generated by its original use as a hematopoietic stem cell marker [26]. CD133 has been identified as a CSC marker in glioblastomas [25] as well as colorectal [4], ovarian [27, 28], hepatocellular [6], lung [8], and pancreatic [29] cancers. CD133 is localized to membrane protrusions and microvilli, but little is known about the function of this protein in cells or CSCs in particular. It is apparent that while CD133 can be used to distinguish CSC populations, it may not play a direct or critical role in cancer formation or CSC maintenance. A study demonstrating this point showed that a $CD133^+$ colon cancer population was able to differentiate and self-renew even when CD133 expression had been knocked down [30]. What is clearer is that CD133 has been positively correlated with poor outcomes for cancer patients. A meta-analysis of 603 gastric cancer patients from 8 different studies revealed that CD133 overexpression was linked to lymph node metastasis, distant metastasis, higher drug resistance, an increased relapse rate, and a lower 5-year survival rate [31]. The widespread presence of CD133 in putative CSC populations across numerous tissues, coupled with the poor prognosis of patients overexpressing CD133, validates this marker as a dependable marker for CSCs as well as a potential cancer drug target.

CD24

Cluster of differentiation (CD24) is yet another surface marker used to demarcate CSC populations. CD24 is a notable CSC marker as both its presence [11] and absence [3, 32] has been used to denote CSC phenotypes depending upon the tissue. CD24 is a surface expressing glycoprotein, also known as heat stable antigen (HSA), which was initially identified as a marker for hematopoietic subpopulations, typically B-cells. Numerous functions have been suggested for this protein, including signaling and

cell attachment, and its expression can be seen in various cell types, most commonly acting as a marker of differentiation for hematopoietic and neuronal stem cells. CD24 is often seen in the context of adaptive immune response in which its expression can be seen in pre or immature-B cell populations or in activated T-cells [33]. The function of CD24 in tumor cells may be explained by the association of the marker with P-selectin, a molecule expressed by platelets and vascular endothelium, which may play an important mechanistic role in cancer cell adhesion and metastasis. In addition to acting as positive or negative marker for CSCs, depending upon the tissue of origin, the expression of CD24 has also been associated with poor prognosis, larger tumors, and lymph node metastasis in a range of cancers, demonstrating its influence on clinical outcomes [34-36].

ESA or epCAM

Epithelial specific antigen (ESA), also known as epithelial cell adhesion molecule (epCAM) has been used to identify CSCs from breast [3], colorectal [37], and pancreatic [11] cancer. As the name implies, ESA is a surface marker typically expressed on epithelial cells, which regulates cell-to-cell adhesion. ESA is overexpressed in a majority of epithelial cancers, such as colorectal cancer, and as a result it has been the subject of numerous studies and targeted chemotherapy strategies. ESA has further been linked to the migratory and invasive capabilities of breast cancer and is highly expressed in breast cancer metastases [38]. By disrupting the expression of ESA, the migration and invasion of cancer cells *in vivo* can be diminished. The upregulation of this transmembrane glycoprotein, as a result, may play a role in the metastatic potential of proposed CSCs.

ALDH activity

Increased aldehyde dehydrogenase (ALDH) activity has been used to identify CSCs with little technical difficulty. ALDH can refer to any number of enzymes classified as aldehyde dehydrogenases which act to catalyze the oxidation of aldehydes entering or produced within the body. By oxidizing aldehydes, these enzymes transform potentially deleterious compounds into carboxylic acids, preparing them for cellular metabolism. In this way they act to detoxify the cell. ALDH enzymes are highly expressed in liver cells, but their expression has also been used to distinguish numerous progenitor cells including hematopoietic stem cells [39] and neural stem cells [40] among others. ALDH enzymes are therefore theorized to contribute to stem cells' robust ability to survive chemical stresses throughout the body. The cytoprotective effect of ALDH enzymes utilized by these stem cell populations, however, can also be used to protect CSCs from chemotherapy treatments.

ALDH activity has been used to identify CSCs of various tissues including colon [41], breast [42], head and neck squamous cell carcinoma [43], ovarian [44], and lung [45]. ALDH1 is commonly proposed to be the source of ALDH activity in CSCs, and its expression has been widely used as a CSC marker. However, unspecific ALDH activity can also be utilized to categorize cells as CSCs using the ALDEFLUOR assay. The ALDEFLUOR assay contains BODIPY-aminoacetaldehyde (BAAA) which enters intact, viable cells and is oxidized by ALDH enzymes producing fluorescent BODIPY-aminoacetate (BAA). This fluorescence can be detected using fluorescent microscopy or flow cytometry. The noncytotoxic nature of ALDEFLUOR additionally enables sorting of live CSC populations via fluorescence-activated cell sorting (FACS). Identification of CSCs using ALDH activity assays is a powerful tool for cancer researchers due to this ability to separate viable subpopulations combined with the association of ALDH activity with MDR.

Hoechst 33342 exclusion

Hoechst 33342 is a stain capable of permeating intact cell membranes, which produces blue fluorescence when bound to nuclear DNA. This property is used to visualize nuclei, similar to 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), while maintaining cell viability. Stem cells and other cells overexpressing drug efflux pumps possess the unique ability to exclude this stain, and as a result Hoechst 33342 exclusion has been used to label various progenitor cells such as hematopoietic stem cells [46]. The drug efflux pumps responsible for Hoechst 33342 dye exclusion may further contribute to MDR in cancer cells. Hoechst 33342 excluding cells, also known as the side population (SP), of tumors have therefore been inves-

tigated as a source of drug resistant CSCs. SP cells have been shown to exhibit stem-like properties in hepatocellular [7], lung [47], ovarian [9], breast [2] and other cancers as well as exhibiting enhanced drug resistance. Like the ALDEFLUOR assay, segregation of hypothesized CSCs using Hoechst 33342 exclusion can be combined with FACS techniques to isolate a viable CSC populations based upon a characteristic associated with MDR.

EMT capability

Epithelial-mesenchymal transition (EMT) is the process undergone by epithelial cells in which the cells alter their morphology, lose their polarity, and break cell-cell or cell-matrix adhesions. In this way, the cells gain mobility and invasive potential. EMT is an essential process during development and wound healing, allowing epithelial cells to produce a population of mobile cells able to migrate to target locations and reestablish basal and apical polarity once there [14]. CSCs are hypothesized to possess enhanced EMT capability, enabling the cells to survive in the absence of cellular adhesion in addition to enhancing their resistance to apoptosis. CSCs having undergone EMT are thought to then reattach and produce metastatic tumors or circulate throughout the body in a dormant state, only to become active years later and cause distant cancer relapse to occur. The ability of CSCs to undergo EMT can be investigated by determining the expression of EMT related proteins such as Twist, Snail, or N-cadherin [48].

More commonly, however, EMT capability is assessed by removing any opportunity for cellular attachment. This can be accomplished through the use of non-adherent well plates, stirred bioreactors, serum-free growth conditions, or encapsulation in hydrogels. When in these conditions, cells without EMT capability will die leaving only cells that have undergone the transition. The remaining cells often grow in what are referred to as tumorspheres which have been shown to be enriched in CSCs in numerous tissues [49-51]. A major drawback of using these selective growth environments is the relatively low purity of CSCs in the resulting population. Further, CSCs within tumorspheres of a large enough size are likely to differentiate into phenotypically diverse cells. Still, drug discovery efforts directed at limiting the EMT capability of CSCs should be encouraged as this ability lies at the heart of the spread and recurrence of cancer that plagues many patients.

Drug resistance in cancer stem cells

While resistance to chemotherapy treatments is not necessary to define CSCs, drug resistance is commonly associated with CSC populations. In fact, when resistance to a drug is induced, an increase in the percentage of cells possessing CSC markers has been observed [52]. Resistance to specific chemotherapy agents in cancer cell lines is typically promoted *in vitro* by exposing the cells to gradually increasing doses of the drug or by exposing the cells to several cycles of clinically relevant chemotherapy doses followed by drug free media to mimic the treatment patients actually receive. The enrichment of CSCs following chemotherapy regimens observed both *in vitro* and in clinical studies [13] has enormous implications on drug discovery efforts and future cancer treatment. Without the ability to target and kill CSCs, chemotherapy treatments will continue to leave patients at risk for tumor recurrence and developed drug resistance. The following proteins and properties of CSC are thought to contribute to drug resistance in CSCs and therefore represent ideal targets for future chemotherapy or chemotherapy sensitizing drug discovery efforts. It is important to note, however, that healthy stem cells share many of the properties imparting drug resistance to CSCs, and as a result targeting these properties may lead to unwanted side-effects on otherwise healthy tissues.

ABC transporters

ATP-binding cassette (ABC) transporters are transmembrane proteins that serve a crucial cytoprotective role for healthy stem cells throughout the body. The function of these proteins is to pump toxic compounds from the cell body before their deleterious effects can occur. These pumps are able to act on a large variety of compounds including many chemotherapeutic agents. The expression of ABC transporters has been used to indicate CSC phenotypes in multiple tissues and also plays a role in developing the multiple drug resistance (MDR) typical of CSCs [53]. Members of the ABC transporter family that appear to be highly expressed

in CSCs include, but are not limited to, ABCB1, ABCG2, and ABCB5 [54]. The expressions of these proteins have been suggested as markers for CSCs, but the lack of appropriate antibodies makes their detection more difficult than previously discussed markers. The ability of the SP to exclude Hoescht 33342 is a result of ABC transporters, specifically ABCG2, making SP isolation an indirect method of CSC isolation based upon ABC transporter expression [54].

Many of the ABC transporter proteins have been "discovered" multiple times in the context of chemotherapy resistance leading to confusion in their identification. For example, ABCG2 is often referred to as breast cancer resistance protein (BCRP) alluding to its ability to confer MDR to breast tumor cells. ABCG2 expression has been identified in the drug resistant subpopulations of many cancer models including K562 chronic myeloid leukemia cells [55] and MCF7 breast adenocarcinoma to name a few [56]. The cell lines in these experiments were made resistant through selection with various chemotherapies such as doxorubicin.

ABCB1 is another ABC transporter with multiple aliases. ABCB1 has been referred to by the names multidrug resistance protein 1 (MDR1), cluster of differentiation 243 (CD243), and most commonly P-glycoprotein 1 (P-gp). ABCB1 contributes to the efflux of many widely used chemotherapeutic agents including anthracyclines, *vinca* alkaloids, and taxanes making it a highly clinically relevant MDR protein [57]. Reduction of the expression of ABCB1 has been shown to lead to an increased chemotherapy sensitivity of colorectal CSCs in addition to MDR cell lines of differing origin [57]. By targeting ABC transporters, the unique resistance of CSCs can theoretically be reversed, sensitizing them to traditional chemotherapy treatments.

ALDH enzymes

Another strategy CSCs employ in order to exhibit MDR is the rapid metabolization of the chemotherapy agents they are subjected to. As mentioned previously, the presence of ALDH enzymes and their activity is a commonly used marker to identify CSCs. ALDH enzymes exert their effect by oxidizing aldehyde groups of drug molecules, preparing them for future cell metabolism and thus detoxifying the cell. ALDH enzymes may also play a role in the differentiation of healthy and malignant stem cells. Inhibition of ALDH activity in $ALDH^{hi}/CD44^{+}$ putative breast CSCs convincingly resulted in a loss of MDR [58]. Interestingly, the inhibition of ALDH activity using diethylaminobenzaldehyde (DEAB) further sensitized these CSCs to radiation therapy. By eliminating ALDH activity from tumors, the breakdown of chemotherapeutic agents within the tumor will be slowed resulting in a more effective treatment. Cytotoxic compounds which do not act as substrates for ALDH enzymes or that reduce their activity may have a unique ability to induce apoptosis in CSCs and act as more effective long-term treatments.

Pro-survival signaling and stem cell maintenance

CSCs hijack many of the pro-survival signaling cascades and maintenance proteins seen in healthy stem cells. In this way, CSCs have a tendency to survive cellular stresses capable of eliminating differentiated cancer cells in a similar fashion to non-malignant stem cells. For example, mechanistic target of rapamycin (mTOR) and signal transducer and activator of transcription 3 (STAT3) play a role in the maintenance and proliferation of healthy and cancer stem cells. The activation of phosphatase and tensin homolog (PTEN) and subsequent inhibition of mTOR and STAT3 results in a significant decrease in CSC viability and overall tumor drug resistance [59].

The stem cell maintenance proteins Wnt, Hedgehog, and Notch are also upregulated in CSCs. These molecules play a major role in maintaining the stem-ness of CSCs and activating the expression of stem cell related transcription factors such as octamer-binding transcription factor (Oct4) and Nanog as well as influencing EMT [60]. Stem cell maintenance proteins such as these ensure CSCs will continue to asymmetrically divide, allowing the CSC phenotype to persist in a number of harsh conditions. Dysregulation of these pathways is hypothesized to promote gradual CSC differentiation leading to decreased tumor viability in response to chemotherapeutics, making them an attractive target for the treatment of both bulk tumors and CSCs.

Quiescence

Cellular quiescence is defined by a reduced occurrence of mitotic divisions within a cell population. Quiescence is recognized as a trait of most somatic stem cells, allowing them to survive in a state of relative dormancy and reduce the accumulation of DNA mutations over time [61]. While debate remains as to whether or not chemotherapy agents have a diminished effect on quiescent cells, experiments on leukemia stem cells have shown that forcing these cells out of their dormant state results in increased drug sensitivity [62]. The hypothesis behind this pathway for MDR is that diminished cellular metabolism, failure to proceed throughout the entirety of the cell cycle, and lack of DNA multiplication allows CSCs to avoid activating the targets of many chemotherapeutic toxins. Quiescence of CSCs not only potentially influences MDR, but also enables CSCs to remain dormant at the site of the original lesion or migrate throughout the body for years before attaching and initiating new tumors. Targeting the quiescence of CSCs has the potential to increase the efficacy of current therapeutic methods against CSCs within the original tumor as well as prevent CSCs from entering dormant states capable of initiating new tumors in patients in remission.

Natural products targeting cancer stem cells

Natural products (NPs) have played an important role in medicine for much of recorded human history. The earliest recorded use of medicinal plants dates back approximately 5000 years to a list of Sumerian drug recipes written on a clay tablet, but there is evidence that Neanderthals may have used plants for medicinal purposes as far back as 60,000 years ago [63, 64]. Even today many people in the world rely on medicinal plants for their healthcare needs. It is estimated that 70-95% of people in most developing countries use traditional medicine for their primary healthcare needs [65]. Traditional Chinese and Ayurvedic medicine have historically served as primary healthcare for many people in developing nations, and both systems have drawn the attention of pharmocognosists from around the world.

Active compounds from various organisms have had great success as pharmaceuticals. This is especially true in the case of cancer chemotherapeutics. Between 1981 and 2006, 63% of anticancer drugs being used came from NPs, were inspired by NPs, or were synthesized from a natural pharmacophore [66]. The most profitable chemotherapy drug in history, taxol (or paclitaxel), is a natural product derived from the bark of the Pacific Yew Tree [67]. Taxol was discovered through a random screening of approximately 15,000 species of plants [43], but targeted screening of known medicinal plants for anticancer properties has also been historically successful. For example, the vinca alkaloids vincristine and vinblastine have been used clinically in cancer therapies for over 50 years [68]. These compounds were isolated from the rosy periwinkle, *Catharanthus roseus,* a plant used in both traditional Chinese medicine and Ayurvedic medicine. Bacteria have also been a source of successful anticancer agents. Anthracyclines, such as doxorubicin, are isolated from certain *Steptomyces* bacteria and have been used to treat breast cancer for decades [69].

With advances in technologies such as high throughput screening (HTS) and combinatorial chemistry in the 90's, the cancer related drug discovery efforts of many pharmaceutical companies shifted to targeted therapies [70]. These targeted, receptor specific therapies relied upon small synthetic molecules or antibodies that could act as "magic bullets" to treat specific cancer cells. Combinatorial chemistry has allowed vast libraries of new chemical entities to be generated synthetically which can be tested against disease related targets. Thousands of compounds from combinatorial chemistry libraries can be analyzed every day using HTS [71]. In addition, advances in proteomics and genomics have enabled researchers to attempt to model molecules that can interact with specific biological targets. The initial success of these targeted therapies including Gleevec and Herceptin led many to believe that traditional NP based drug discovery had become obsolete [45].

However, the limited number of successful drug candidates from targeted therapies, the relatively small number of cancers successfully treated with new therapies, and the higher risk of cancer developing a resistance to treatment created a renewed interest in natural product drug discovery in the late 2000's [46]. The limited efficacy of targeted therapies is of in-

creased likelihood in CSCs, due to the lack of agreed upon universal CSC markers and the many survival mechanisms which they employ. Numerous NPs and their derivatives have shown early clinical success or have received FDA approval for the treatment of cancer since the recent renewal in their interest [46, 72]. Despite the obstacles facing the screening of NPs using HTS, they have shown many advantages over synthetic chemical entities. Natural products are thought to possess "privileged structures" that are specialized to interact with biological targets allowing them to influence multiple cellular pathways simultaneously. This ability is crucial in combatting cancer and CSCs, as the robust survivability of cancer is often the result of many different mechanisms. Additionally, the chemical character and diversity of NPs is more favorable than that of synthetic molecules. When compared to synthetic libraries, NP libraries tend to have more chiral centers, higher steric complexity, fewer heavy atoms, more solvated hydrogen-bond donors and acceptors, and a larger variety of molecular properties [48]. Furthermore, historic use of a medicinal plant from which a NP is isolated can speak to the safety of compound for human consumption and the potential to limit side-effects.

The continued ability of natural compounds to compete with synthetic chemical entities has shown that NP based drug discovery is still relevant and capable of advancing the treatment of cancer. It is likely that the successful screening of NPs for cancer killing potential can be successfully applied to screening for CSC targeting agents. A few promising NPs have been utilized to target CSCs *in vivo* and *in vitro*. **Figure 1** depicts the role that such NPs may play in preventing cancer metastasis and recurrence. These compounds may have the potential to sensitize CSCs to conventional treatments, directly induce cell death in CSCs, force CSCs to differentiate, or prevent CSCs from entering a dormant and more resistant state. A brief review of these compounds can be found below. The reader of this review is directed to other reviews for a more comprehensive list of NPs capable of targeting CSCs [12, 73, 74].

Polyphenols

Many natural products used as pharmaceuticals can be classified as polyphenols. Polyphenols are structurally defined by the presence of aromatic benzene rings bonded to hydroxyl groups, but they encompass a number of structurally diverse compounds. These subgroups include flavonoids, stilbenes, tannins, lignans, and phenolic acids among others. Polyphenols of various groups have been demonstrated to regulate inflammation, angiogenesis, cell growth, invasiveness, and apoptosis *in vitro* [75]. As a result, they have been studied extensively in the context of cancer prevention and metastasis. Recently, these investigations have been extended to determine the effect of polyphenols on CSCs. The polyphenols resveratrol and curcumin are notable examples of NPs that have been shown to exhibit cytotoxic effects on CSCs.

Resveratrol: Resveratrol is a polyphenolic stilbene derivative most commonly found in the skin of grapes and berries. It has undergone extensive examination for its anti-inflammatory and antioxidant properties in addition to many other useful biological properties. These attributes give resveratrol the attractive potential to act as a cancer chemopreventative. Resveratrol has been shown to induce apoptosis and promote S-phase arrest of select cancer cells. This potential was demonstrated in Hep G2 hepatocyte carcinoma cells in vivo at concentrations ranging from 10 to 50 μM [76]. At concentrations higher than 50 μM, however, resveratrol induced G_1/G_0 arrest which was confirmed in a separate study using a number of ovarian cancer cell lines [76, 77]. Resveratrol has further been shown to induce cell death through a non-apoptotic mechanism at concentrations between 50 and 100 μM in a ovarian cancer cell lines [77]. This variety of mechanisms demonstrates the ability of resveratrol, like other NPs, to influence numerous biological mechanisms simultaneously making it an attractive anticancer agent.

Resveratrol may also be able to eliminate CSC populations from tumors. The compound has been shown in a study by Shankar et al to induce caspase-3/7 activated apoptosis in $CD44^+/CD24^+/ESA^+$ pancreatic CSCs at 10 to 30 μM concentrations. The study also found that 10 to 20 μM resveratrol was able to inhibit both stem cell maintaining factors, such as Nanog and Oct-4, as well as anti-apoptosis proteins of the Bcl-2 family in the pancreatic CSCs. Additionally, EMT proteins, such as Snail and

Slug, as well as the EMT capability of the pancreatic CSCs in non-adherent conditions was inhibited in response to 10 to 20 μM of resveratrol. Further, the expression of the drug efflux pump ABCG2 was inhibited after administration of 10 to 30 μM of resveratrol, potentially sensitizing the cells to conventional chemotherapy treatments. The apparent ability of resveratrol to target CSCs and act as a chemopreventative and anti-inflammatory drug was further demonstrated using a mouse tumor model. The frequency of tumor formation in $Kras^{G12D}$ mice, spontaneous pancreatic tumor forming mutants, was significantly diminished when treated with resveratrol for 10 months [78]. The ability of resveratrol to induce apoptosis in CSCs as well as reduce their tumorigenic potential in vivo was additionally supported in a $CD24^-/CD44^+/ESA^+$ model of breast cancer stem cells. In this study, apoptosis was induced in the breast CSCs through a FAS mediated pathway after incubation with 50 or 100 μM resveratrol. The tumorigenic potential of the cancer stem cells was significantly diminished in female nude mice through the administration of either an oral gavage or intraperitoneal injection of 22.4 kg/body weight of resveratrol, giving significant evidence that resveratrol is able to disrupt tumor formation by targeting CSCs [79].

While resveratrol exhibits extremely promising anticancer effects in preclinical studies in vivo and in vitro, resveratrol has failed to translate this success to clinical trials. This is due, in large part, to extremely low bioavailability, high effective dosages, and the rapid metabolism of resveratrol to glucuronide, sulfate, and hydroxylate conjugates [80, 81]. These conjugates, once absorbed into the bloodstream fail to provide the same health benefits as free resveratrol. As a result, there have been efforts to engineer resveratrol formulations or drug delivery systems aimed at increasing the bioavailability of resveratrol. These include formulations to stabilize resveratrol in the body, formulations to increase the aqueous solubility of resveratrol, and encapsulation of resveratrol in various lipids, micelles, or polymer structures with the aim of sustained, concentrated, and/or targeted release [80, 81].

Curcumin: Curcumin is another polyphenol which has been thoroughly investigated for its anticancer properties. This compound is a major component of turmeric, a spice widely used in Indian and many Middle-Eastern cuisines. Curcumin has been shown to exhibit an anti-inflammatory effect and promote apoptosis in cancer cells [82]. It has been used in clinical trials demonstrating its safety at high doses and activity against pancreatic neoplasms in human patients despite its low bioavailability [83]. The antitumor properties demonstrated by curcumin have led to investigations of its potential to target CSCs.

Curcumin has been used to inhibit the formation of breast cancer mammospheres in vitro by 50% and 100% using 5 μM and 10 μM concentrations, respectively, demonstrating the ability of curcumin to inhibit CSC's ability to undergo EMT [84]. An analogue of curcumin, GO-Y030, was demonstrated to induce apoptosis, diminish tumorsphere formation, and inhibit STAT3 phosphorylation in $ALDH^+/CD133^+$ colon CSCs when used at 2 to 5 μM concentrations. The ability of this analogue to target tumor initiating cells was further demonstrated using a NOD/SCID mouse model. When given a 50 mg/kg intraperitoneal injection of GO-Y030, the average tumor weight resulting from a xenograft implantation of 1×10^5 CSCs was diminished by 58.10% [85]. Curcumin has also been suggested as a supplement to current chemotherapy treatments. Curcumin in combination with FOLFOX, a commonly prescribed combination of leucovorin calcium, fluorouracil, and oxaliplatin, was able to decrease the viability and diminish EMT of colon CSCs to a higher extent than FOLFOX alone [86].

While curcumin shows great potential as an anticancer agent and has been used in a number of clinical trials against cancer, it suffers similar shortcoming to resveratrol. Namely, the rapid metabolism and excretion of curcumin, along with its hydrophobicity, results in low bioavailability which has been demonstrated using mouse models [87, 88]. Numerous drug delivery studies have been conducted to increase the bioavailability of curcumin including the use of adjuvants to interfere with metabolism, encapsulation in liposomes and nanoparticles, and the use of more stable structural analogues [89].

Flavonoids

Flavonoids are a major class of polyphenolic secondary metabolites found in numerous

medicinal plants. They are derived from flavone which contains two phenyl rings and one heterocyclic ring. Flavonoids are commonly found compounds throughout the plant kingdom, and as a result, they are widespread throughout the human diet. Due to their abundance in fruits, vegetables, nuts, spices, and herbs, a flavonoid rich diet has been suggested as a feasible means of cancer chemoprevention [90]. Certain flavonoids including, quercetin and kaempferol, have been implicated as apoptosis inducers, antioxidants, inflammation regulators, and angiogenesis inhibitors. Further, certain flavonoids have been shown to have an effect on heat shock proteins, multiple drug resistance, adhesion, metastasis, and angiogenesis [91]. The high number of CSC related properties which seem to be affected by flavonoids have led to their investigation as CSC targeting agents. A review of one such flavonoid, quercetin, is presented below.

Quercetin: Quercetin is a flavonol secondary metabolite found throughout many species of plants. Quercetin is a known anti-inflammatory agent and anti-oxidant which has been demonstrated to induce programmed cell death in many malignant cancer cell lines. Quercetin has been shown to interfere with a number of cellular pathways associated with the formation and maintenance of human cancers including down regulating P53, inhibiting tyrosine kinase, inhibiting heat shock proteins, and inducing type II estrogen receptor expression [92]. Quercetin has further drawn attention as a potential CSC targeting therapeutic.

Not only has quercetin been shown to inhibit the proliferation of $CD133^+$ colon CSCs at a concentration of 75 μM, but it also increases the sensitivity of these cells to doxorubicin (Adriamycin). In fact, when combined with 50 μM quercetin, doxorubicin doses were more effective at inhibiting CSC proliferation *in vitro* than doxorubicin doses three times more concentrated but lacking quercetin [93]. This finding demonstrates the potential of quercetin and other natural products to enhance the use of other chemotherapeutics to eliminate CSC populations. The use of lower doses of chemotherapeutic agents in combination with natural products such as quercetin may result in diminished off target toxicity while also inducing apoptosis in CSCs, improving patient outcomes, lowering the risk of cancer recurrence, and preventing metastasis formation.

Other CSC models have been targeted using quercetin including $CD44^+/CD133^+$ prostate CSCs. At a concentration of 20 μM, quercetin lowers the viability of prostate tumor spheroids grown in non-adherent flasks as well as diminish the migratory, invasive, and colony forming potential of $CD44^+/CD133^+$ prostate CSCs [94]. In this same publication, quercetin was shown to synergize with epigallocatechin gallate, a catechin found in tea, synergistically amplifying the above effects on these prostate CSCs. As is the case with many other NP's, however, quercetin's poor solubility, poor permeability, and instability result in diminished bioavailability [95]. The relatively high dose of quercetin required to elicit a biological response in combination with these issues warrant further drug delivery efforts to increase the lifetime and concentration of the compound at the site of the neoplasm.

Alkaloids

Alkaloids are a class of pharmacologically active organic compounds distinguished by the presence of nitrogen and aromatic rings in the chemical structure. Alkaloids are produced throughout the plant kingdom, but are usually found in higher plants [96]. Many alkaloids have been used throughout history in the medical field from quinine for the treatment of malaria to vinblastine for the treatment of multiple carcinomas. Several alkaloids have been used clinically in the treatment of cancer with great success, demonstrating their importance in the field. A small group of alkaloid compounds have even been shown to differentiate between healthy and cancerous DNA, inhibiting *in vitro* cancer DNA synthesis while leaving healthy DNA unaffected and resulting in a potential cancer treatment with diminished side-effects [97]. New investigations on alkaloids are still being conducted showing further antineoplastic, anti-metastatic, and MDR inhibiting potential [76]. These results suggest a potential for alkaloids to eliminate CSCs, and indeed, a number of compounds belonging to the alkaloid family have been shown to target CSCs *in vitro* and *in vivo*. Three promising anti-CSC alkaloids, dihydrocapsaicin, piperine, and berberine, are presented in the following sections.

Dihydrocapsaicin: Capsaicin is the secondary metabolite and alkaloid responsible for the hotness of many species of pepper. Dihydrocapsaicin (DHC), a saturated derivative of this compound, has exhibited numerous anti-neoplastic properties. DHC has been shown to induce dose-dependent and catalase regulated autophagic cell death in colon and breast cancer cells when used at concentrations between 50 and 400 μM [98]. However, when autophagic cell death was inhibited through treatment with the inhibitor 3-methyladenine, DHC instead induced caspase-3 activated apoptosis in these cell lines. Further, when apoptosis was inhibited by the addition of peptide zVAD, autophagic cell death was enhanced. This ability to promote separate modes of cell death is a useful tool in targeting CSCs due to the many cell death evading pathways active in CSCs. This ability further highlights the potential of NPs to influence multiple cellular mechanisms and produce a robust cytotoxic effect on cancer cells.

A review of CSC related patents revealed that DHC is further hypothesized to exhibit a cytotoxic effect on neural CSCs [73]. In one of the patents collected in the review, US20090076-019A1, a neurosphere assay was invented to screen potential drugs for activity against neural stem cells. As the percentage of putative CSCs are increased in cancer neurospheres, compounds capable of inducing cell death in these spheres can be thought of as agents targeting neural CSCs. DHC was identified in this patent as one of several lead compounds which showed an ability to target $CD133^+$ neural CSCs. The high IC_{50} values of DHC, however, limit its use as an effective chemotherapeutic agent, especially when one considers the low bioavailability common for many NPs. Further research is warranted to determine if DHC or an analogue can target any phenotype of CSCs with higher efficacy than what has been shown.

Piperine: Piperine is a promising antineoplastic alkaloid found in black and long pepper. The use of piperine has previously been suggested as a cancer chemopreventative, but it has also demonstrated the ability to induce cell cycle arrest, endoplasmic reticulum stress, and apoptosis when exposed to colon cancer *in vivo* at concentrations between 75 and 150 μM [99]. The treatment of colon cancer cells with piperine has been shown to reduce the ability of the cells to form non-adherent spheres and colonies, suggesting the inhibiting effect of piperine on CSCs. The apoptotic effect of piperine has additionally been confirmed using prostate cancer cells [100].

The ability of piperine to target stem cells specifically has been investigated in a breast tissue model. After pre-treatment with 5 to 10 μM piperine, the mammosphere formation potential, ALDH expression, and Wnt signaling of unsorted breast tissue was significantly diminished [84]. Interestingly, the differentiated population of these cells was seemingly unaffected by the piperine treatment. The potential of piperine to target CSCs without affecting other cells is a fantastic example of the robust ability of NPs to influence molecular pathways while imparting only benign side effects. Piperine has additionally been suggested for use in combination therapies with compounds, such as resveratrol or curcumin, due to its ability to inhibit metabolic pathways. By slowing the glucuronidation of these compounds, piperine inhibits the metabolism and clearing of NPs and increases their bioavailability [101]. By inducing a cytotoxic effect on CSCs and increasing the efficacy of other compounds, piperine acts as an ideal complementary medication to other NP chemotherapies.

Berberine: Berberine is a tetracyclic, isoquinoline alkaloid found in the roots and stems of numerous plants. Berberine producing medicinal plants have been used as anti-inflammatories in Ayurvedic medicine for years, and the compound has been shown to induce dose-dependent apoptosis, initiated by reactive oxygen species generation, in a broad spectrum of cancers [102, 103]. The apoptosis induced by berberine goes through an internal caspase-9 dependent pathway which results in a loss of mitochondrial membrane integrity. Like many natural products, the bioavailability of berberine is low in the body, limiting the potential of berberine as a drug. This obstacle is being overcome through the use of targeting liposomes as a drug delivery system [104]. This delivery system encapsulated berberine into liposomes which were engineered to deliver the compound directly to the mitochondria of $CD44^+/CD24^-$ breast cancer stem cells. Using this system, 1-50 μM of berberine was able to produce dose-dependent apoptosis in breast CSCs. The drug was further able to induce the expression

of the pro-apoptotic protein Bax and activate caspase-9 and caspase-3 leading to apoptosis in CSCs isolated from MCF-7 mammospheres.

Additionally, berberine has been used to inhibit the expression of ABC transporters responsible for MDR in CSCs [78]. Diminishing MDR, especially in CSC populations, makes berberine an attractive complementary medicine when currently accepted cytotoxic agents are unable to kill cancerous cells. An *in vivo* mouse model in which MCF-7 breast CSCs were injected into female nude mice followed by an array of berberine treatments and formulations demonstrated this synergistic capability. A mixture of 10 mg/kg of berberine liposomes and 10 mg/kg of paclitaxel liposomes was able to reduce the average tumor size in these mice by 85.5% compared to the control after just 21 days [104]. In this way, berberine could be used to either target CSCs alone or in combination with traditional chemotherapy agents.

Other

Many other natural compounds which do not fit into the classifications of polyphenols, flavanoids, or alkaloids have shown promise in targeting CSCs. Retinoids are an example of these compounds. Vitamin A, also known as retinol, generates a number of biologically active retinoids, including All-Trans Retinoic Acid (ATRA). ATRA has found clinical success in the treatment of acute promyelocytic leukemia under the trade name Tretinoin. The drug is marked by its successful induction of remission coupled with relatively mild side effects [105]. The mechanism of action utilized by ATRA is through induction of cellular differentiation of leukemic and hematopoietic cells, and this differentiation induction has further been observed in other types of stem cells [106]. The differentiation potential of retinoids presents a unique potential for cancer treatment, namely differentiating CSCs into a cell population more sensitive to classic chemotherapeutic regimens. Additionally, ATRA acts as an inhibitor of ALDH activity, potentially reversing a cause of MDR in CSCs [58]. ATRA has thus been used to limit the tumorsphere formation ability and CSC percentage of breast cancer cells *in vivo* [107].

The lactone antibiotic brefeldin A is another NP that cannot be classified as a polyphenol, flavonoid, or alkaloid. It has shown anticancer potential in a number of cancer types including leukemia, colon, and prostate through p53 independent mechanisms [108, 109]. Brefeldin A is produced by certain fungal organisms and acts as a protein transport inhibitor, preventing proteins from traveling from the endoplasmic reticulum (ER) to the Golgi apparatus. Subsequently, brefeldin A initiates ER stress, potentially leading to its apoptotic effects. Recently, brefeldin A has been shown to preferentially induce cell death in suspension cultures over adherent cultures of the human breast adenocarcinoma line MDA-MB-231. In the same publication, brefeldin A also down-regulated the expression of CD44, reduced the ability of the cells to form colonies in soft agarose, and reversed the EMT [110]. Preferential killing of putative CSCs and inhibition of colony forming potential was similarly reported in the human colorectal cancer line Colo 205 [111]. This preferential killing has the potential to diminish CSC populations while limiting the side effects typically associated with chemotherapy.

Conclusion

The cancer stem cell hypothesis, while still being investigated, presents explanations to many of the issues facing cancer treatment today. The CSC hypothesis explains the mechanisms underlying cancer recurrence, metastasis, and, to a degree, multiple drug resistance. Cancer treatments directed toward the eradication of CSCs could lead to higher survival rates and brighter prognoses for patients who fear cancer regression could occur at any time. Current cancer treatments are insufficient in regard to the eradication of CSC populations, likely due to the multitude of survival mechanisms utilized by CSCs and the lack of definitive, universal, single molecule targets that separate CSCs from healthy stem or somatic cells. Natural products have historically been an excellent source of bioactive compounds capable of targeting multiple pathways, and current investigations are underway to screen NPs for their effect on the CSC population of numerous cancer types. Many different NPs have exhibited a range of CSC inhibitory properties, and it is likely that more have yet to be discovered. As a result, NPs should continue to be screened as potential chemotherapy agents, complimentary treatments for compounds already in clinical use, and cancer prevention

molecules with special attention focused on their ability to target CSCs. Further, due to the limited bioavailability and rapid metabolism of many NPs, these drug discovery efforts must be coupled with continued efforts to engineer robust drug formulations and delivery systems.

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Disclosure of conflict of interest

None.

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