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## It's Getting Hot in Here: Targeting Cancer Stem-like Cells with Hyperthermia

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### Abstract

Cancer stem-like cells (CSCs) are a subset of cancer cells that are resistant to conventional radiotherapy and chemotherapy. As such, CSCs have been recognized as playing a large role in tumor initiation and recurrence. Although hyperthermia is broadly used in cancer treatment either alone or in combination with radio- or chemo-therapy, its potential to target CSCs is not well understood. In this review, we discuss different types of hyperthermia and potential mechanisms of action in cancer treatment, particularly in regards to killing CSCs.

### Keywords

Hyperthermia; Cancer stem-like cells; Chemotherapy; Cytotoxic treatment; Radiation; Nanoparticle; Ablation; LITT

### Introduction

Despite advances in understanding the molecular changes underpinning cancer and improved technology and treatments, cancer remains a leading cause of death in America.

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### Conflict of Interest

Authors declare no conflict of interest.

The cancer stem-like cell (CSC) hypothesis posits that a subset of tumor cells have a high capacity for self-renewal, have the ability to differentiate into multiple lineages and can give rise to tumors [1–4]. These CSCs are highly malignant and can persist or proliferate in spite of cytotoxic treatment [1–4]. Therefore, these CSCs play a large role in tumor progression. Development of new treatment modalities that are able to target and kill CSCs may provide more durable cancer control [1–4].

Hyperthermia is a potent radiosensitizer that has been shown in numerous clinical trials to improve tumor control. Importantly, the efficacy of hyperthermia is seen across many cancer types, including breast cancer, prostate cancer, melanoma, sarcoma, rectal cancer, bladder cancer, esophageal cancer, cervical cancer and glioblastoma suggesting that it has broad clinical applicability [5–24]. Recently, combined hyperthermia and radiation has also been shown to improve pain palliation in patients with bone metastases compared to radiation alone [25]. Therefore, hyperthermia has widespread usage for patients with both locoregional disease and advanced cancers and can be used for patients with a variety of cancer types. The value of hyperthermia as a treatment has in fact been observed for centuries. Hippocrates, the father of modern medicine, is known to have said, “Those who cannot be cured by medicine can be cured by surgery. Those who cannot be cured by surgery can be cured by heat. Those who cannot be cured by heat, they are indeed incurable”. Over the years, medicine and surgery have seen significant advances, and hyperthermia fell by the wayside. However, in modern times, hyperthermia is making a resurgence due to improved technology in delivering hyperthermia and in non-invasive thermometry techniques.

Hyperthermia is classified into two broad categories based on the target heating temperature. Thermal ablation refers to treatments with target temperatures above 50°C and mild temperature hyperthermia refers to treatments with temperatures between 39 and 43°C [26]. While thermal ablation largely kills tumor cells due to the direct cytotoxic effects of heat, mild temperature hyperthermia uses heat as an adjunct treatment to enhance the cytotoxic effects of radiation and chemotherapy [26–28]. The biologic effects of thermal therapy are dependent on time and temperature. The mechanisms underlying the biologic effects are multi-factorial and impact the tumor population itself, the tumor microenvironment and immune system.

## Methods for Administering Hyperthermia

Radio-frequency hyperthermia is the most widely used hyperthermia technique worldwide and is typically used for ablative heating [28–30]. To achieve heating, radio-frequency electrodes are passed into the tumor tissue under image guidance. A high-frequency alternating current is then passed through the electrodes to cause the rapid oscillation of ions in nearby cells, resulting in frictional heating [27,31]. The range of heating is limited to the millimeter range because it relies on heated tissue to conduct current to surrounding areas [32]. The short range of heating also limits the ability to heat tumors near blood vessels because the heat is dissipated too quickly [32,33].

Microwave hyperthermia is an alternate method of delivery that can overcome some of the limitations of radio-frequency hyperthermia. Microwave heating uses waves of higher

frequency to kill cells. Unlike radio-frequency thermal therapy, microwave hyperthermia does not pass an electrical current through tissue, but rather creates an oscillating electromagnetic field that forces ions and dipoles to align with the field, causing them to rotate as the field oscillates [31,32,34]. This rotation causes friction that heats the tissue. Microwave hyperthermia presents several advantages compared to radio-frequency hyperthermia. While radio-frequency hyperthermia relies on ions inside tissue to conduct current, microwave hyperthermia creates an electric field, the effective range of which is larger without risking damage to tissue closer to the antenna or probe [32]. Microwave hyperthermia has a much higher effective range of up to 3 cm [32].

Laser interstitial thermal therapy (LITT) is a relatively new method of administering hyperthermia that uses a stereotactically placed laser probe to heat surrounding tissue with a low power (10–15 Watts) infrared laser (at Nd-YAG range) [35,36]. Heat essentially is produced after absorption of laser in the tissue and transferred up to 1.5–2 cm from the laser probe by conduction. To control the extent of thermal ablation, a specific sequence of MRI (MR-thermometry) is used to measure relative changes of temperature within the magnetic field. For deep seated lesions, including brain tumors, LITT is used in conjunction with MR-thermometry to give accurate thermal ablation of the target lesion [35,36]. The minimally invasive nature of LITT under MR-thermometry guidance has permitted the expansion of hyperthermia to deep and difficult to access tumors including intracranial and retroperitoneal tumors [35,36].

High intensity focused ultrasound (HIFU) (also called focused ultrasound surgery (FUS)) utilizes an ultrasound beam with very high energy to increase the temperature rapidly in the target tissues [37–39]. A single HIFU exposure usually treats a very small volume along the ultrasound axis. Multiple exposures can be used side by side to achieve coverage of a large volume of tumor [37–39]. One advantage of HIFU is that it creates a steep temperature gradient in a small focused area and effectively creates a sharp boundary of damage in the target tissue while sparing adjacent normal tissues [37–39].

Nanoparticles can also be used to augment heating within a tissue when exposed to electromagnetic energy [40–42]. These particles include magnetic nanoparticles (such as iron oxide), gold-silica nanoshells, solid gold nanoparticles and carbon nanotubes [40,41]. The outer shell of nanoparticles can be modified molecularly to facilitate their dissemination and uptake by specific cell types, including tumor cells [42]. Additionally, nanoparticles may be loaded with cargo including cytotoxic drugs or oncolytic viruses that can be released upon disruption by a heat source [43]. Nanoparticles can be administered systemically to exploit the leaky vasculature of primary tumors to enhance intra-tumoral delivery [44]. However, nanoparticles often display a patchy, near perivascular deposition within the well-vascularized regions of tumors [45]. Some blood vessels such as those associated with brain tumors are not as leaky as blood vessels found in other solid tumors. While the blood-brain barrier is partially breached in regions with glioma cells, the ‘compromised’ blood-brain barrier still presents a major challenge, especially in hypoxic and avascular regions of glioma dispersion [46]. Since high spatial concentrations of nanoparticles are required for hyperthermia, direct intratumoral delivery of 12 nm magnetic nanoparticles has been used in clinical trials for hyperthermia treatment of prostate tumors and recurrent glioblastoma

[47,48]. When subjected to an external alternating magnetic field, the nanoparticles vibrate and heat up to kill surrounding cells. Because non-ionizing electromagnetic radiation can be applied remotely to heat the nanoparticles, this technology is considered noninvasive but requires good visualization of the target tumor [42,49]. A typical drawback of the application of iron oxide nanoparticles is associated with the indefinite exclusion of MRI for subsequent monitoring of tumor progression after initial injection of nanoparticles and the residual MR signals that interfere with follow-up MR imaging.

## Hyperthermia in Cancer Therapy

When hyperthermia is applied to a tumor, three different reaction zones can be distinguished based on the temperature and duration of heating: a central zone that is directly and immediately beyond the application site, a peripheral zone that is around the central zone and is heated to a lower temperature, and an outer region which is not directly affected by the heat [28,42,50]. Hyperthermia causes cellular injury directly and indirectly in these different zones via different mechanisms, although some overlap may exist. The extent and type of cellular damage varies as a function of temperature and time. A high temperature for a short period of time can achieve similar levels of cell kill as lower temperature heating for a longer period of time.

### Direct Effects to Tumor Cells

Hyperthermia causes membrane dysfunction to contribute to cell death. Rising temperature affects the stability fluidity and permeability of cellular membranes, including the plasma membrane, mitochondrial membranes, and other cytosolic membranes [50,51]. These membrane changes can compromise the function of transmembrane transport proteins, ion channels, cell surface receptors and other membrane-associated proteins and disrupt lipid rafts or signal transduction hubs [50,51]. However, the degree of membrane dysfunction strongly influences cell fate. For example, some changes in membrane potential, intracellular sodium and calcium content do not correlate well with the rate of cell death [52–54]. On the other hand, mitochondrial dysfunction induced by hyperthermia can lead to cell death [50,55].

Another direct effect of heat is the denaturation of proteins, especially under high temperatures. Denaturation and inactivation of these proteins can impact a broad range of cellular processes including cellular metabolism, protein synthesis, nucleic acid synthesis and DNA/RNA polymerization [50,56]. After mild hyperthermia, some cellular functions can recover. Proteins may refold, and RNA and protein synthesis may recover. However, DNA replication and repair typically remain repressed [57]. This is thought to be due to the aggregation of denatured proteins in the nuclear matrix and irreversible changes to chromatin structure that impair DNA synthesis and repair [57]. Hyperthermia can inhibit the function of DNA-polymerases- $\alpha$  and  $-\beta$  and can also facilitate degradation of the DNA repair protein BRCA2 to inhibit homologous recombination [57,58]. Hyperthermia itself is believed not to cause severe DNA damage, but rather indirectly contributes to DNA damage by reducing the efficiency of the DNA damage repair machinery [59].

Hyperthermia can induce cell death by necrosis and apoptosis. The cells in central application zones, which are confronted with high temperatures, usually die by necrosis. However, some subpopulations of cells may escape immediate hyperthermic killing. These resistant cells in the central zone and the cells in the peripheral zone, which receive lower temperature hyperthermia, may die within hours of heat cessation [50,51]. Mild temperature hyperthermia can induce apoptosis through both the intrinsic and extrinsic pathways. Hyperthermia can activate Caspase-2 which then binds to the adaptor protein RAIDD to cleave and activate Bim, which promotes mitochondria-dependent apoptosis [60]. Hyperthermia can also activate Bim to induce Caspase-2 independent apoptosis. In addition, hyperthermia can activate Fas, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to trigger the extrinsic apoptotic pathway [61,62]. Additionally, heat treatment can increase the production of reactive oxygen species (ROS) by activation of xanthine oxidase and/or facilitating mitochondria respiration to produce  $O_2^-$ . Moreover, hyperthermia negatively affects SOD1 expression and the enzymatic activities of SOD1 and SOD2, whereas it is able to activate NADPH oxidase [63–65]. This increase in ROS can facilitate apoptosis [66,67].

### Alterations to the Tumor Microenvironment

Hyperthermia can also modify the tumor microenvironment to modulate tumor growth and recurrence. Hyperthermia is well known to increase perfusion within tissue and compromise the integrity of blood vessels [33,68–70]. Hyperthermia can damage endothelial cells, alter the adhesiveness of the vessel wall, and increase the leakiness of blood vessels and viscosity of blood [68]. These changes in perfusion can influence local pH, oxygen and nutrient supply in the tumor, rendering them more stressed and more susceptible to cytotoxic therapy [71]. Hyperthermia can also improve tumor oxygenation, making cells more susceptible to radiation, and may improve the penetration of chemotherapy into the tumors [69,72].

Local hyperthermia can also affect the immune system and augment the anti-tumor immune response. With increased perfusion, hyperthermia can increase immune cell penetration into the tumor [73,74]. Hyperthermia-mediated tumor cell death releases the intracellular contents of tumors including RNA, DNA, metabolites and proteins that are immunogenic [75]. The tumor-specific molecules are taken up by dendritic cells and macrophages and trigger specific anti-tumor innate and adapted immune responses. Hyperthermia also promotes the production and release of pro-inflammatory cytokines from tumor cells and increases serum levels of interleukin- $1\beta$  (IL- $1\beta$ ), IL-6, IL-8 and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) [76–78]. Induction of heat shock proteins (HSPs) can also enhance anti-tumor immunity. Extracellular HSPs function as chaperones to bind antigens released by dying tumor cells. The protein complexes are recognized and internalized by antigen-presenting cells (APCs) [79–81]. HSPs also activate NK cells, T-lymphocyte and macrophages to provide a multi-pronged anti-tumor response [80–82].

### Thermotolerance

When tumor cells are exposed to sublethal thermal treatment, they can develop tolerance to sequential thermal stress. This phenomenon is called thermotolerance [83]. Numerous biochemical and molecular changes are involved in this process. Thermotolerance may be

due to the accumulation of heat shock proteins (HSPs), which are preferentially upregulated by transcriptional and translational mechanisms upon hyperthermic stress [83]. Hsp90, Hsp70, Hsp60, and Hsp27 families play a critical role in thermotolerance by maintaining protein structure, preventing protein aggregation and facilitating re-folding of proteins under heat stress [83,84]. HSPs also regulate apoptosis through caspase dependent and independent pathways [85]. Hsp27 and Hsp70 can also inhibit the translocation of pro-apoptotic factor Bid to mitochondria. Hsp70 can inhibit the cleavage and activation of Bid to repress mitochondria-mediated apoptosis [86,87]. HSPs can also suppress Fas, TNF and TRAIL-mediated apoptosis through binding to and inhibiting Daxx and ASK-1 or modulating IKK complex stability and activity. Hyperthermia can also affect other signaling pathways, including Integrin-linked kinase, JNK and p38 MAPK kinase activities that contribute to thermotolerance [86,87]. While thermotolerance is seen in vitro, its impact on clinical efficacy of hyperthermia is controversial.

### Side Effects of Hyperthermia

In addition to thermotolerance, another factor to be considered during hyperthermia is its potential side effects. These adverse effects are mainly due to normal cell damage and are dependent on the area being treated. In whole-body hyperthermia, which has been used to treat metastatic cancers with chemotherapy in clinical trials, whole body hyperthermia can cause side effects including diarrhea, nausea, vomiting and more rarely results in cardiac and vascular dysfunction [6,88]. In regional and local hyperthermia, normal cells in the proximity of the targeted cancer cells can be damaged by heat. Limitations in directing heat to the tumor volume or suboptimal thermometry in the heated area can cause local areas of heat deposition that can damage normal tissue [6,88,89]. These hyperthermia-associated side effects include tissue swelling, erythema, blistering of skin, or burning pain in the heated volume, in the case of superficial hyperthermia. Most of these side effects are reversible and heal after conservative measures [6,9,88,89]. Side effects of hyperthermia for deeper tissue heating are dependent on the organ being treated. For example, patients undergoing hyperthermia treatment for locally advanced prostate cancer may develop mild urinary symptoms due to swelling of the prostate gland and mild proctitis [19,20]. This can be mitigated with a short course of steroids. Deep tissue hyperthermia can also increase sweating and vasodilation, causing a drop in blood pressure and contributing to postural hypotension [90]. The utilization of specific hyperthermia methods may also minimize side effects. For example, HIFU causes a very steep temperature gradient and damages tumor cells in a small area around the probe. Cancer biomarker-based nanoparticles selectively deliver heat to cancer cells. Overall, hyperthermia is relatively safe when administered properly, and as seen in many clinical trials, offers clinical tumor control benefits that exceed the side effects of hyperthermia [6].

### The Effects of Hyperthermia on Cancer Stem-Like Cells

Cancer stem-like cells (CSCs) are a rare population of cancer cells that can self-renew and differentiate into progeny with limited proliferative potential. CSCs sit at the apex of hierarchically organized tumors. CSCs have strong tumorigenic activity compared to non-stem cancer cells and can establish an entire tumor [1,3,56]. CSCs usually reside in specific

niches that orchestrate their fate. Niche components that support the undifferentiated state of CSCs include communication with contacting cells such as other stromal cells and endothelial cells, extracellular matrix components, soluble factors including Wnt, TGF $\beta$  and other cytokines, and physical states such as hypoxia and low pH [91,92].

CSCs are resistant to ionizing radiation and chemotherapy. CSCs preferentially activate the DNA damage checkpoint in response to radiation and repair DNA damage more efficiently than non-CSC cancer cells [93]. Inhibition of DNA damage checkpoint kinases Chk1 and Chk2 sensitizes CSCs to radiation [93]. CSCs can also overexpress ROS scavenger proteins to limit the amount of DNA damage after radiotherapy, which is largely mediated by ROS. Pharmacological depletion of ROS scavengers can improve the radiosensitivity of CSCs [94].

CSCs can develop resistance to chemotherapy via different mechanisms, including enhancing efflux of chemotherapy from tumor cells or interfering with chemotherapy metabolism. CSCs can overexpress ABC transporters to pump out chemotherapy [95,96]. CSCs can express enzymes to inactivate drugs, for example, aldehyde dehydrogenase (ALDH) which inactivates cyclophosphamide, temozolomide and doxorubicin [97]. CSCs can also epigenetically silence the enzymes that are needed to catalyze prodrugs into their active forms [97]. Additionally, CSCs can augment pro-survival signaling pathways, including upregulating BCL2 family proteins and activating Notch and Wnt pathways, to protect themselves from the cytotoxic effects of chemotherapy [98-101]. Some CSCs can also exit the cell cycle and remain in a quiescent state, reducing their sensitivity to chemotherapy and radiation, which preferentially targets rapidly proliferating cells [102,103].

The CSC niche also plays a major role in therapeutic resistance. The hypoxic regions where many CSCs reside are devoid of functional blood vessels and chemotherapy is unable to penetrate to these areas in appreciable concentrations. Furthermore, low ROS levels in these areas render CSCs more resistant to free radical damage induced by radiation and some chemotherapies [104,105]. Other components of the CSC niche including the extracellular matrix, cancer-associated fibroblasts and immune cells may provide signaling stimuli including hepatocyte growth factor (HGF), interleukin 6 (IL-6), fibroblast growth factor (FGF), neuregulin 1 to further facilitate therapeutic resistance [106,107].

Based on its effects on CSCs and their microenvironment, hyperthermia may have the potential to eliminate cancer stem cells and sensitize them to radiotherapy and chemotherapy. First, hyperthermia can denature proteins that pump out or inactivate chemotherapy. Second, hyperthermia can impede DNA damage repair pathways and thus sensitize CSCs to radiation and some types of chemotherapy. Third, hyperthermia can kill tumor cells independent of their cell cycle status, permitting killing of quiescent CSCs. Fourth, heat treatment can increase perfusion inside the tumor and so reduce hypoxia and alter ROS levels that support CSCs. Alternatively, hyperthermia can also cause collapse of blood vessels and damage endothelial cells to reduce blood flow to create local pockets of hypoxia. Therefore, hyperthermia can have complex effects within the tumor and in particular, CSCs within the perivascular or hypoxic niches. In addition, hyperthermia can

enhance the anti-tumor immune response to target CSCs. This can be mediated in part by increasing cell kill, exposing CSC-specific antigens to APCs and improving immune cell recruitment to the tumor.

Although hyperthermia is broadly used in cancer treatment alone or in combination with surgery, radiotherapy and chemotherapy, whether and how hyperthermia can help to eliminate chemo- and radio-resistant CSCs has not been extensively studied. Some studies suggest that CSCs may be more sensitive to hyperthermia than normal stem/progenitor cells [108]. Wierenga, et al. compared the sensitivity of normal and acute myeloid leukemia (AML) stem cells to mild temperature hyperthermia (43°C). While heat treatment had only a mild effect on normal stem and progenitor cells, it greatly reduced AML stem cell survival and leukemia initiation ability [108]. This suggests that hyperthermia can be used therapeutically to purge leukemic stem cells with minimal adverse effects on normal stem/progenitor cells.

However, in some other tumors, hyperthermia alone may have little effect or even negative effects on CSCs in terms of cell kill. In a model of breast cancer, breast CSCs can survive better than bulk breast cancer cells to water bath hyperthermia, and the CD44<sup>high</sup>/CD24<sup>low</sup> stem cell fraction in bulk breast cancer cells was increased after hyperthermia [109]. This increased survival of breast CSCs was attributed to overexpression of Hsp90. Interestingly, the addition of an Hsp90 inhibitor sensitized breast CSCs but not bulk cancer cells to hyperthermia, suggesting that under thermal stress, breast CSCs are dependent on Hsp90 for survival and that combining Hsp90 inhibition with hyperthermia can target the breast CSC population [109].

However, hyperthermia mediated by nanoparticles may overcome CSC resistance to conventional heat treatment. Burke, et al. showed significantly reduced tumor size and prolonged mouse survival when treated with multi-walled carbon nanotubes, which were heated by near-infrared radiation [109]. Breast CSCs, as well as bulk tumor cells, died from necrosis in their study. In another report, superparamagnetic iron oxide nanoparticles have been used to induce magnetic hyperthermia in A549 lung cancer and MDA-MB-231 breast cancer cell lines [110]. This magnetic hyperthermia compromised stem-cell properties, including reduced side population phenotype, mammosphere formation ability, ALDH activity and tumor initiation ability [110]. In contrast, conventional water bath thermal treatment was less efficacious [110]. Notably, magnetic nanoparticle based hyperthermia promoted higher ROS generation for a prolonged time compared to conventional hyperthermia [110], suggesting the method of hyperthermia delivery can influence the extent of cell death.

Hyperthermia has also been combined with radiotherapy, chemotherapy or antibody-based targeting technology to improve CSC elimination. Atkinson, et al. reported that optically activated gold nanoshell-based hyperthermia sensitized breast cancer stem cells to radiation and reduced the percentage of Lin<sup>-</sup>CD29<sup>H</sup>CD24<sup>H</sup> CSC subpopulation, which are resistant to radiation [111]. Hyperthermia reduced DNA damage repair in breast CSCs, rendering them vulnerable to radiation-induced DNA damage [111]. Similarly, in glioblastoma, hyperthermia alone had no appreciable effect on the survival or proliferation of glioma CSCs



[112]. However, when combined with radiation, hyperthermia preferentially reduced glioma CSC self-renewal, viability and proliferation. This was mediated by suppression of radiation-induced AKT signaling and impaired DNA damage response. Combined hyperthermia, delivered by a microwave applicator, and radiation reduced tumor growth and extended mouse survival in a CSC-derived mouse model of glioblastoma [112].

Hyperthermia can also induce metabolic stress and enhance the effects of chemotherapy. This strategy has been used to sensitize CSCs to metformin, a diabetes drug that also has anticancer activity [113]. Thermal therapy can activate AMPK and suppress the mTOR/S6K pathway to enhance the cytotoxic effects of metformin [113]. Wierenga, et al. also reported that hyperthermia can improve the leukemic stem cell killing mediated by ET-18-OCH<sub>3</sub>, an anti-tumor lipid chemotherapy that is toxic to leukemic cells in a temperature-dependent manner [108].

The combination of hyperthermia treatment with CSC targeting technology shows promise. Single-walled carbon nanotubes (SWNTs) conjugated with CD133 monoclonal antibody (anti-CD133) were used to target glioma stem cells, which frequently express the cell surface marker CD133 [114]. Upon nanotube internalization and heating with a near infrared laser, CD133<sup>+</sup> but not CD133<sup>-</sup> cells were killed. Additionally, heating significantly suppressed the tumor initiation ability of CD133<sup>+</sup> cells [114]. These data suggest that hyperthermia combined with antibody-based strategies can be used to kill CSCs efficiently.

## Conclusion

The use of hyperthermia to treat cancer has a long history. Hyperthermia has consistently improved the efficacy of radiotherapy and chemotherapy for many types of cancers. The CSC model sheds light on another potential therapeutic benefit of hyperthermia. Strategies that combine hyperthermia with cytotoxic agents, metabolic stressors or immune therapies may improve CSC kill by targeting the cancer cells themselves and modulating their microenvironment. The method of administering heat may also influence cell kill. More work is needed to define the optimal modes of hyperthermia to kill CSCs safely and efficiently. The combination of hyperthermia and immunotherapy to target CSCs also holds great potential, and further studies are needed to understand how best to integrate hyperthermia with immuno-oncology. Heat therapy was recognized for its therapeutic effects by ancient physicians. It is once again emerging as an important treatment modality that fights cancer through multiple mechanisms.

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