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Hyperthermia can alter tumor physiology and improve chemo- and radio-therapy efficacy

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ABSTRACT

Hyperthermia has demonstrated clinical success in improving the efficacy of both chemo- and radio-therapy in solid tumors. Pre-clinical and clinical research studies have demonstrated that targeted hyperthermia can increase tumor blood flow and increase the perfused fraction of the tumor in a temperature and time dependent manner. Changes in tumor blood circulation can produce significant physiological changes including enhanced vascular permeability, increased oxygenation, decreased interstitial fluid pressure, and reestablishment of normal physiological pH conditions. These alterations in tumor physiology can positively impact both small molecule and nanomedicine chemotherapy accumulation and distribution within the tumor, as well as the fraction of the tumor susceptible to radiation therapy. Hyperthermia can trigger drug release from thermosensitive formulations and further improve the accumulation, distribution, and efficacy of chemotherapy.

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1. Introduction

Over the course of evolution, humans have developed very effective temperature regulation mechanisms in order to maintain an environment that allows cells to prosper [1,2]. Hyperthermia (HT) treatment involves elevating *in vivo* tissue temperature above the normal, closely controlled physiological range. In contrast to other thermal therapies such as ablation [3] and cryotherapy [4] that exert direct cytotoxic effects by inducing large changes in temperature, HT mildly increases temperatures within the body to between ~39 – 45 °C. HT can be applied to the whole body [5] or target specific regions, often a single tissue [6]. Many different techniques are used in order to induce HT temperatures. In the clinical setting, local HT is delivered by external energy sources including ultrasound (US) [7], radiofrequency [8], and microwave [9]. These same techniques can be employed in the pre-clinical research environment, although the most common approach involves submerging the animal's limb in water [10]. Target tissue temperatures must be accurately monitored as innate thermoregulation mechanisms will dissipate energy and reduce temperature. Whole body HT is commonly achieved by elevating ambient temperature with lower temperatures (i.e. 39.5-40.5 °C) and longer durations (i.e. 6-12 h) compared to locally targeted HT [11].

This article focuses on the use of HT in oncology as an adjuvant to chemotherapy (ChT) and radiotherapy (RT). There is solid clinical evidence to support the addition of HT to ChT and RT treatment protocols in order to improve patient outcomes. In 2018, Issels et al. reported Phase III (NCT00003052) clinical trial results that represent the most comprehensive study demonstrating the enhancement of standard ChT by addition of HT [12]. Patients with localized, high-risk soft tissue sarcomas received four cycles of neoadjuvant ChT (doxorubicin, ifosfamide, and etoposide) or ChT and HT (42 °C, 1 h). Almost all patients then underwent surgical resection and the majority of patients in both treatment groups received post-surgical RT (50 – 60 Gy). The addition of HT into the treatment regimen significantly increased median survival time from 6.2 to 15.4 years. Ten-year survival rates were 52.6% for 162 patients treated with ChT and HT and 42.7% for 167 patients treated with ChT alone. In 2016, Datta et al. published two landmark systematic reviews establishing the therapeutic benefit of adding HT to RT in breast [13] and cervical [14] cancers. Eight studies of locoregional recurrent breast cancer involving 627 patients compared therapeutic outcomes of RT with HT to RT alone. A statistically significant difference was observed between treatment groups with complete response observed in 60.2% of patients receiving RT + HT compared to 38.1% for RT alone. Six previously reported clinical trials were analyzed in which patients with locally advanced cervical cancer received either RT + HT (n = 215) or RT alone (n = 212). Patients treated with RT and HT were more likely to demonstrate complete response compared to RT alone, but the 8.4% increase in survival observed in the RT + HT group was not significant. These promising results warrant further study on the benefits of combining HT with both ChT and RT. Selection of the right patient population is critical to the success of clinical trials. These trials included patients with locally advanced or recurrent tumors that are well suited to the potential therapeutic enhancement of HT. This article discusses how tumor physiology and microenvironment can further be used to stratify patients most appropriate for HT in order to increase the probability of clinical trial success.

The recent clinical advancement of HT is rooted in many years of research. In 1974, Robinson et al. published their observation that HT significantly increases the effect of RT on tumors and importantly, does so to a greater extent than sensitization of normal skin tissue [15]. Following this, a number of studies published in the latter half of the 1970s confirmed that HT was able to directly kill cancer cells as well as sensitize cells to the toxic effects of ChT and RT [16–20]. Fig. 1 depicts the logarithmic increase in published cancer research over the past sixty years (red) as well as the increasing fraction of these publications that involve HT (black). The shape of the curve is reminiscent of the Gartner hype cycle [21] with clear peaks of interest in the early 1980s and 1990s. The two most highly cited papers preceding the initial peak are Weinstein et al. "Liposomes and local hyperthermia: selective delivery of methotrexate to heated tumors" [22] and Overgaard's "Simultaneous and sequential hyperthermia and radiation treatment of an experimental tumor and its surrounding normal tissue *in vivo*" [23]. These events are referred to as technology triggers in the Gartner hype cycle and represent seminal *in vivo* papers demonstrating the ability of HT to increase liposome drug accumulation in the tumor [22] and the time and temperature dependent manner in which HT can improve RT efficacy [23]. Along with the second era of hype in the early 1990s that saw highly cited improvements in heating technology, clinical advancement, and a broadening of research efforts [24–28], these early discoveries laid the foundation for the current sustained 'plateau of productivity'.

In order to exert their intended effect, intravenously administered drugs must be transported from the vascular space through the vascular walls into the interstitial tumor space and ultimately to their specific target that often lies within the cancer cells. A variety of drug delivery strategies including advanced formulations and physiological modifications have been developed to increase the efficiency of this process [30]. HT can induce physiological changes and trigger drug release in order to impact the accumulation and distribution of drugs within the tumor.

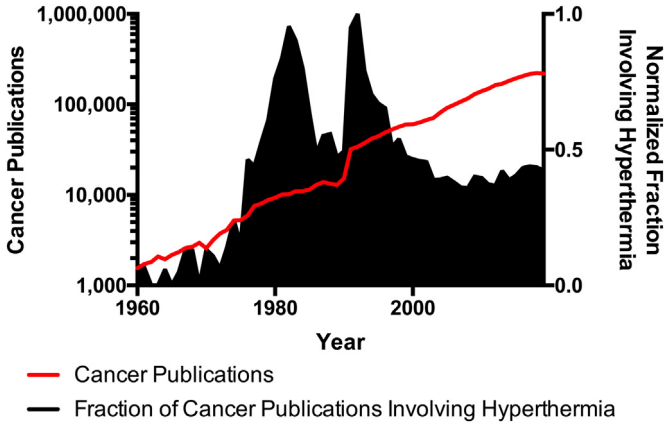

Fig. 1. Frequency distribution of cancer research publications involving HT over a fifty-year period demonstrating the Gartner hype cycle. Data sourced from a Web of Science database search for articles published between 1960 – 2019 that include 'cancer', 'tumor', or 'tumour' within the title, abstract, and keywords. Represented in black are the percentage of those publications that contained 'hyperthermia' within the title, abstract, or keywords, normalized to 1 for the highest year (1992).

Convection and diffusion are the transport phenomena that govern delivery of drugs from the vasculature to the tumor as well as penetration into the tumor tissue [31]. Drug diffusion is a concentration-dependent process that follows Fick's first law. Diffusion is approximately inversely proportional to molecular weight, but is also dependent on numerous factors including molecular shape and solute—solvent interactions [32]. Diffusion-driven processes contribute to accumulation of small molecule drugs to a much greater extent than is the case for macromolecular or nanomedicine drugs [33]. Indeed, one analysis estimated that it would take 100 nm liposomes 84 d to diffuse through 100 μm of tumor tissue, whereas free doxorubicin would diffuse the same distance in 5 s [34]. Furthermore, the diffusivity of drugs is dependent on the bulk media. Drugs will more readily diffuse from blood into perivascular spaces compared to diffusion within the stroma-rich tumor microenvironment [35]. In the context of drug delivery, convection is the bulk movement of fluid that transports drugs and other molecules [36]. Fluid flows along pressure gradients and the heterogeneous distribution of fluid pressure within the tumor results in regions of high and low convection-mediated drug delivery [37]. In general, fluid pressures are lower at the tumor periphery, resulting in preferential accumulation of nanomedicines compared to the centre of the tumor [38]. However, even minimal transvascular pressure gradients can result in significant accumulation of nanomedicines [36,39]. Therefore, temporal fluctuations in either vascular or interstitial fluid pressure can strongly influence drug accumulation. The multifactorial impact of HT on the transport of chemotherapeutic agents from the vasculature into the tumor interstitium is discussed throughout this review.

This review specifically focuses on the physiological response to HT, in particular changes in blood flow and vascular permeability that impact the tumor microenvironment, in particular hydrogen ion concentration (pH), partial pressure of oxygen (pO_2), and interstitial fluid pressure (IFP). The influence of these changes on the efficacy of ChT and RT are discussed in addition to the measurement of each parameter in the pre-clinical and clinical context. Specific attention is paid to the use of triggered release drug formulations, particularly thermosensitive liposomes (TSL) in combination with HT. As outlined in Fig. 2, the impact of HT on the efficacy of ChT and RT is a complex, multifactorial process that involves several different physiological pathways. Without clearly defined conditions, generalizations about whether or not a specific intervention exerts a certain effect are often of little value in scientific research. HT has the potential to exert the effects depicted in Fig. 2, but these outcomes are dependent on the manner in which HT is delivered (e.g. duration and temperature) and the underlying tumor biology. Furthermore, HT exerts several impacts that are outside the scope of this review, including its effects on the immune system and effects at the molecular biology level such as the heat shock protein response and the impact on DNA damage repair. These responses can also impact ChT and RT efficacy and are discussed thoroughly in other articles within this special issue. While much is known, there is still a great deal of research to be conducted in order to optimize the clinical integration of HT with ChT and RT. Personalized characterization of tumor physiology including blood flow parameters, pO_2, pH, and IFP could identify patients that are more likely to benefit from HT and further increase the success of future clinical trials.

2. Blood Flow

The most significant physiological response to HT is an increase in blood flow. All of the other physiological responses discussed in this review are a direct result of an increase in blood flow within the tumor volume. When heat is applied to many human and animal tissues, blood flow rapidly increases in order to dissipate heat [40]. This phenomenon is most prominent in tissues such as the skin, while the cooling effect is less effective in tissue such as the testes [41]. The Pennes bioheat Equation (1) was developed to model this heat dissipation in biological tissues [42].

$$\nabla \cdot k\nabla T + \dot{q}_m + \rho_b c_b \omega_b (T_b - T) = \rho c \frac{\partial T}{\partial t} \quad (1)$$

The change in the temperature of the tissue as a function of time ($\partial T/\partial t$) is dependent on the three main factors, which are included on the left hand side of Equation (1). With regards to HT applied to a tumor, the first term ($\nabla \cdot k \nabla T$) describes heat dissipated out of the tumor and into the surrounding tissue by conduction, with k being the thermal conductivity of tissue. The second term ($\dot{q}_m$) denotes heat added to the tumor that is generated by cellular metabolism. The third term on the left describes the heating or cooling of the tissue resulting from blood flow (ω_b) and is proportional to density (ρ) and specific heat (c) of blood and tissue, the latter two terms remaining relatively unchanged. Under hyperthermic conditions, the tissue has been heated and its temperature (T) is greater than the temperature of blood flowing through it (T_b). As a result, this term is negative, indicating a cooling effect resulting from blood flow. Blood flow (ω_b) is the most easily modulated parameter and increasing blood flow is therefore the most effective method for regulation of tumor temperature following the application of HT. Due to the unique biology of tumors, their physiological response to HT is not the same as most other normal tissues [43]. Much is known on this topic as many of the studies that established the modern discipline of HT research were focused on the impact of HT on blood flow, specifically tumor blood flow [44].

2.1. Effect of HT on tumor blood flow

Dr. Chang-won Song and colleagues were responsible for much of the pioneering work in determining the effects of HT on tumor blood flow. Prior to this, Dr. Song's work during the 1960s and 1970s focused on the impact of RT on tumor blood flow [45,46]. Many of the techniques employed were similar and the RT and HT fields continue to be closely aligned. In 1980, along with Drs. Rhee, Kang, and Levitt, Dr. Song published six research articles studying the relationship between HT and tumor blood flow [47–52]. One study used radiolabelled microspheres and radioscintigraphic quantification to determine the effect of 60 min of water bath heating at 43 °C on several vascular parameters in subcutaneously implanted mammary carcinomas [51]. Significant increases in vascular volume, vascular permeability, and blood flow were observed in skin and muscle tissue, but none of these parameters were significantly altered in heated compared to unheated tumors. It is important to note that both before and after heating, blood flow in

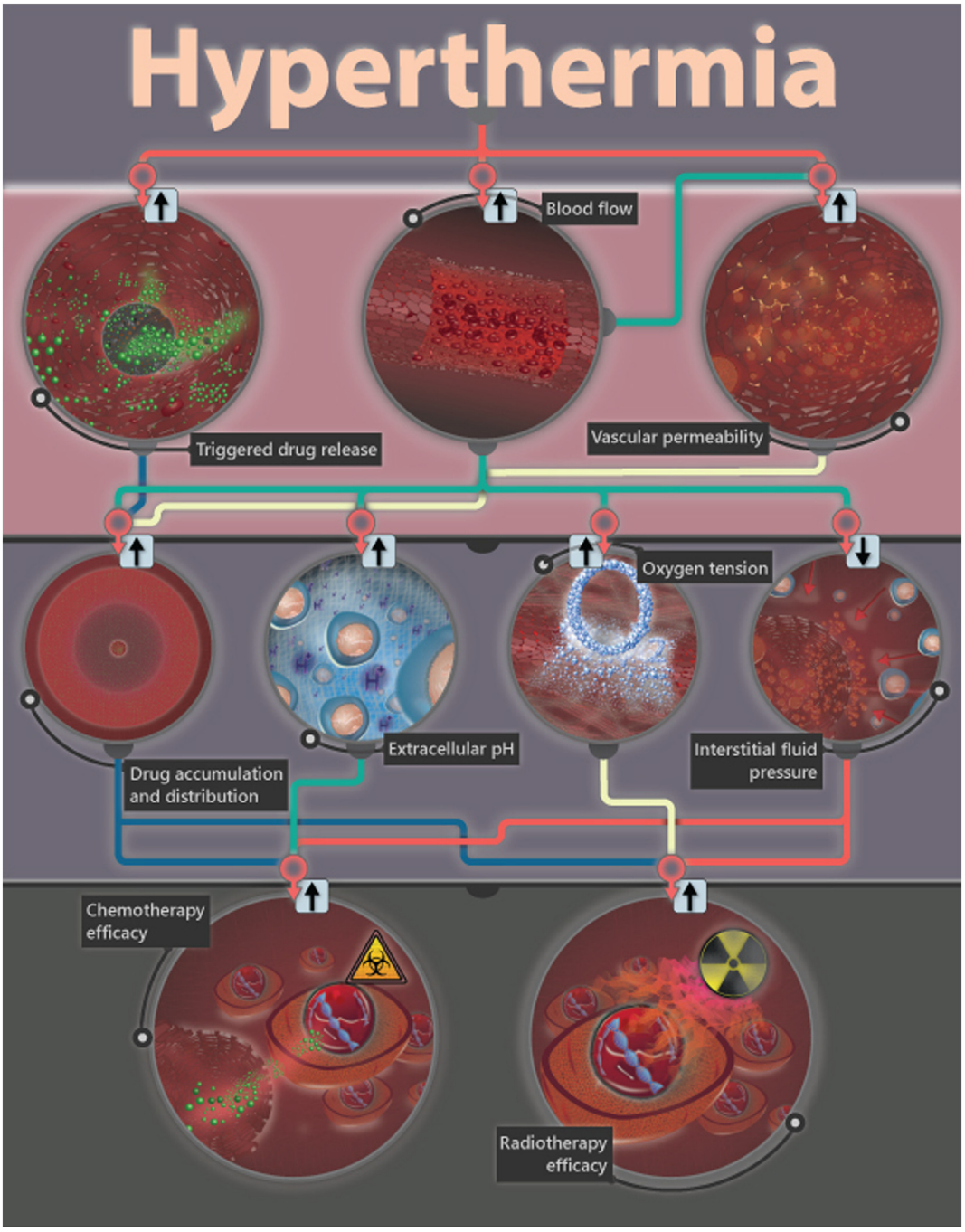

Fig. 2. Potential impacts of HT and the ensuing effects on the efficacy of ChT and RT. This flowchart has been adapted from [29].

normal skin and normal muscle was significantly lower than blood flow in tumors 300 – 700 mg in size. Therefore, even without the increase in blood flow, heat dissipation in these tumors would have been greater compared to normal tissues. Conversely, in many studies blood flow in tumors is less than that observed in normal tissues [53]. Follow-up work demonstrated that this HT regimen induced a marked decrease in vascular volume at 7 and 20 h post-heating suggesting that this HT protocol resulted in vascular damage in this tumor model [52]. In 1984, Jain and Ward-Hartley reviewed the literature to determine the effect of HT on blood flow [53]. Summarizing 28 studies, the authors provided prescient commentary on the heterogeneity associated with blood perfusion of tumors and identified the limitations associated with the macroscopic measurement techniques utilized in most studies. While they noted this further complicates comparison of studies that used a variety of techniques to assess blood flow, the general trend seemed to be that heating to lower temperatures (i.e. < 43 °C) resulted in an increase in tumor blood flow [54,55] while heating to higher temperatures resulted in unchanged or decreased blood flow [56–58]. However, there were also examples wherein heating for 1 h at 42 °C did not result in an increase in blood flow [59]. In a clinically relevant canine study, Vujaskovic et al. employed MW HT for 1 h and measured tumor perfusion by MR imaging before and 24 h following treatment [60]. They separated tumors into two groups: those for which a median temperature of <44 °C was reached during heating and tumors reaching a temperature of >44 °C. Tumor perfusion was significantly higher compared to baseline in the tumors heated to temperatures of <44 °C while perfusion was lower compared to baseline, but not significantly so, in the second treatment group. A recent estimate suggested that

elevating tumor temperatures to levels below ~42 °C is essential to increasing blood flow [61]. However, both time and temperature of HT exposure critically impacts physiological effects. Cumulative equivalent minutes at 43 °C (CEM43) is the standardized measure for quantifying thermal dose within the hyperthermic oncology field and is expertly discussed elsewhere in this issue [62]. Formally proposed by Sapareto and Dewey [63], CEM43 is based on the biphasic slope of the time and temperature dependent Arrhenius cytotoxicity plot [18,64,65]. As this approach is based on cell killing, specific applicability to modern studies focused on modifying tumor physiology and biology has been debated [66–69]. However, it remains clear that exposure time and temperature are critical factors that dictate the impact of HT and must be afforded consideration.

There are several mechanisms by which blood flow within a tissue can increase: an increase in vascular volume within the tissue (either by dilation of blood vessels, re-perfusion of non-flowing blood vessels, or formation of new vasculature) or an increase in the blood flow velocity. Dewhirst et al. were the first to utilize window chamber models and microscopy techniques to directly measure vasodilation during HT [70]. The authors noted varying degrees of blood vessel dilation with smaller vessels seeming to dilate more than larger vessels (e.g. 40 μm diameter vessel increasing to 95 μm upon heating to 42 °C). HT was also able to increase the velocity at which blood was flowing with red blood cell velocity increasing threefold in some vessels, presumably resulting from blood vessel dilation and an increase in blood volume within the tumor [71]. This work also noted the negative effect on blood flow of pre-cooling the tissue to 30 °C prior to administering HT. In vessels cooled to 30 °C prior to administering HT, blood flow was reduced to 5% of that measured in 38 °C control vessels. HT increased blood flow in both cooled and normothermic vessels, but flow in pre-cooled vessels never exceeded 5% of that recorded in non-cooled vessels. This observation is of critical importance given that in preclinical research, animal body temperatures may drop under anesthesia during experimental setup if care is not taken to ensure the animal is kept warm. Furthermore, differences in blood flow may arise from variations in tissue temperature associated with the location of the tumor. More superficial tumors (i.e. subcutaneous) are likely to have lower tissue temperatures compared to more deeply implanted tumors (i.e. intramuscular, some orthotopic sites) and will cool faster under anesthesia. The site of implantation of a tumor can have a profound effect on tumor development including its microenvironment composition, degree of invasiveness, and metastatic potential [72,73]. This is relevant to work in the HT field as many preclinical heating techniques (e.g. water bath) require superficial tumors. Dewhirst et al. also noted that vascular stasis occurred at lower temperatures in tumor arterioles compared to tumor venules and was dependent on the rate of heating, but generally occurred around 42 °C [70]. Rate of heating is seldom reported in research studies that measure the effect of HT on tumor blood flow. The authors hypothesized that HT-induced temporary vascular shunting may be responsible for vascular stasis in venules at lower temperatures [70,74]. Normal tissues produce nitric oxide (NO) in response to stimuli including low pH and low pO_2 [75]. As will be discussed later in this review, these are conditions that tend to occur in tissues lacking in blood flow. NO is known to regulate blood flow by stimulating vessel dilation by activation of soluble guanylate cyclase [76]. Griffin et al. demonstrated that application of HT (i.e. 41.5 – 42.5 °C, 30 – 60 min) was able to increase NO levels in tumors for up to 24 h following treatment [77].

It is apparent that it cannot be unequivocally stated that HT increases tumor blood flow. However, it is equally clear that HT has the potential to increase tumor blood flow. It is widely accepted that vascularity varies substantially between tumors (both of the same and different subtypes) as well as within individual tumors [78]. Heterogeneity of tumor vasculature plays a central role in complicating the prediction of the effect of HT on blood flow [79]. Kelleher et al. used laser Doppler flowmetry to characterize the variable effect of HT on tumor blood flow in DS-sarcomas implanted subcutaneously in rats [79,80]. Tumors were heated at a rate of 0.5 °C / min up to 44 °C. They noted that smaller tumors (i.e. ~700 mm^3) had higher initial blood flow and were less susceptible to vascular stasis as heating commenced compared to larger tumors (i.e. ~2500 mm^3). However, high variability in blood flow within individual tumors was observed in both large and small tumors. In some regions, blood flow increased 2.3-fold whereas in other regions blood flow decreased by 75%. Overall, it can be concluded that HT in the range of 40 – 42 °C has the general effect of increasing tumor blood flow [81]. However, given that tumor blood flow is so temporally and spatially heterogeneous, it is essential that accurate methods are used to measure tumor blood flow in order to determine the effects of HT.

2.2. Effect of RT on blood flow

Blood flow itself does not directly impact the efficacy of RT, but rather mediates treatment outcomes through downstream effects on pO_2, pH, and IFP as will be discussed later. However, RT can directly affect blood flow. Similar to HT, RT affects blood flow in a dose dependent manner. Park et al. completed an exhaustive review of the literature regarding the vascular effects of RT and concluded that tumor blood flow is unchanged or slightly increased during the first several fractions of RT before decreasing following a greater number of treatments [82]. In a clinical study, a dose of 2 Gy/fraction administered five days per week was noted to have increased blood flow for the first two weeks and then subsequently resulted in decreased blood flow during the last 2 – 3 weeks of treatment [83]. A more recent analysis of preclinical studies from Arnold et al. confirms that higher doses of RT have anti-vascular effects while lower dose single fractions can promote vascular growth. They cite numerous studies in which single fractions > ~10 Gy reduce tumor blood flow [84–86] as well as other studies with single fractions within the range of ~2 – 5 Gy in which tumor blood flow was unchanged or increased [87–89]. The effect of RT on tumor blood flow is a dose dependent phenomenon and RT protocols should be selected carefully in order to synergize with other treatment modalities. Indeed, Hu et al. treated mice bearing FaDu tumors with weekly single fractions of 7.5, 9, or 13.5 Gy/d and measured tumor perfusion and oxygenation during and after RT [90]. Only the highest dose of 13.5 Gy increased tumor oxygenation, which was particularly significant given that they also noted that increases in tumor oxygenation following RT were associated with improved local control.

2.3. Effects of blood flow on RT and ChT

ChT remains a standard modality in the treatment of cancer. However, the efficacy of ChT is often limited by heterogeneous distribution of drug within the tumor. For anti-cancer drug therapies to be effective, they must access and kill almost all cancer cells within the tumor [33]. Transport of drugs in the extravascular space of tumors is extremely limited, so drug distribution profiles within tumors tend to be very similar to blood perfusion profiles [91]. This is particularly true for small molecule ChT (i.e. MW < 1 kDa) for which extravascular transport is governed by diffusion [33]. Provided that appropriate pressure gradients exist, fluid convection drives larger molecular weight drugs and nanomedicines into and through the tumor interstitium. Delivery efficiency of these therapies is dependent on perfusion, but also vascular permeability and IFP [92]. These effects will be discussed in greater detail later in this review. Similarly, the effect of blood flow on the efficacy of RT is indirect, being dictated largely by oxygenation levels and will be discussed in Section 2.

While they did not use medical imaging to monitor changes in blood flow in real time, Sun et al. employed immunohistochemical staining to ascertain that HT can increase perfusion in HT29 human colorectal adenocarcinomas in mice [93]. Tumors that had been heated had a higher fraction of perfused vessels compared to tumors that had not. The authors noted variable response to heat within different regions of the same tumor. However, they also noted that the increased accumulation

of the small molecule perfusion marker in the tumor highlights the potential for HT to increase drug delivery. Ausmus et al. measured the effect of HT on blood flow and cisplatin accumulation in mammary adenocarcinomas implanted in rats and heated at 43 °C for 1 h using a Nd:YAG laser [94]. Blood flow was measured using radiolabelled microspheres and was highest following HT and quickly returned to baseline levels. Five separate treatment groups were compared with cisplatin being administered without HT, one hour prior to HT, at the start of HT, at the end of HT, and 1 h post-HT. The highest concentration of cisplatin in tumors was obtained when the drug was administered at the beginning of HT. Given the rapid systemic clearance of cisplatin ($t_{1/2\alpha}$ = 1.5 min; $t_{1/2\beta}$ = 14.5 min in rats) [95] and the short-lived effects of this HT treatment on blood flow it is not surprising that the optimal dosing schedule involves administration of drug concurrently with HT. Furthermore, Landon et al. have demonstrated that HT increases cisplatin uptake into cancer cells via the copper transporter Ctr1 [96].

At lower temperatures (e.g. < 43 °C), HT can increase tumor blood flow while higher temperatures often result in a reduction in blood flow.

3. Vascular Permeability

The presence of drug in blood (determined by the pharmacokinetics of the drug) and the presence of active blood vessels in tumors (i.e. perfusion) are the two most important factors governing drug delivery to tumors. Once drugs reach the blood pool within the tumor, vascular permeability is a critical factor determining transport into the tumor interstitium. Vascular permeability is subdivided into two distinct mechanisms by which compounds transit from the vasculature into the interstitium: transcellular and paracellular transport [97] (Fig. 3). Small molecules drugs (i.e. < ~1000 Da) are transported through endothelial cells via tubular vesicles, called vesicular-vacuolar organelles (VVO) in a process known as transcytosis [98]. Transcytosis is primarily mediated by histamine and vascular endothelial growth factor (VEGF) [99], an important signalling protein regulating the growth of new blood vessels that was originally denoted vascular permeability factor (VPF) [100]. VEGF is known to be overexpressed in many cancers, particularly those low with low pO_2 [101]. Larger molecules are generally thought to travel between adjoining endothelial cells in a process termed paracellular transport. Endothelial cells lining the vasculature are connected by adherens junctions (AJ) and tight junctions (TJ) that maintain vessel integrity [102]. Pericytes regulate vascular permeability by controlling TJ formation and maintaining these junctions [103]. Pericyte abnormalities in tumors likely contribute to increased vascular permeability [104–106]. Pericyte activity is largely controlled by endothelial cell paracrine signaling and the family of angiopoietin signaling molecules that control vascular permeability and angiogenesis through the endothelial receptor tyrosine kinase Tie2 [107,108]. Vascular endothelial cadherin (VE-cadherin) is the most common AJ protein and is therefore critical in the regulation of vascular permeability [109,110]. VEGF is able to disrupt VE-cadherin [111] and thus increase gaps between endothelial cells. VEGF is therefore an important molecule controlling both major types of vascular permeability. As most tumors overexpress VEGF, tumor vasculature is commonly more permeable to both small molecule drugs and nanomedicines compared to normal tissue. Importantly, vascular permeability has been shown to play a central role in cancer metastasis [112]. HT-mediated changes in vascular permeability are dependent on tumor biology. Chen et al. demonstrated this by comparing the effect of a standardized HT protocol (i.e. 42 °C, 1 h) in two separate mouse dorsal skinfold chamber models [113]. In human pharyngeal FaDu tumors, HT resulted in a 9-fold increase in vascular permeability. However, in murine 4T07 breast tumors, no increase was observed. This study clearly demonstrates the importance of considering tumor biology when designing the study and highlights difficulties with comparing studies and generalizing results.

It has long been presumed that paracellular drug transport was responsible for nanomedicine accumulation within tumors, recent reports have suggested that larger drugs such as nanomedicines are also able to accumulate via transcytosis [98,114]. While further study in this area is sure to be done, well-controlled studies of nanomedicine accumulation are able to demonstrate preferential uptake in tumors as a result of functional vascular permeability that leads to therapeutic efficacy. It is important to note that increased accumulation of large molecules and nanomedicines is not a confirmation of increased paracellular vascular permeability. Other factors such as tumor blood supply (e.g. blood volume, blood flow, arteriovenous shunting) and tumor microenvironment factors (e.g. stromal content, IFP) must be controlled and measured in order to accurately attribute the correct contribution to each aspect [33]. In an extensive preclinical study by Wong et al. tumor accumulation of small molecule and nanomedicine contrast agents was used to study the relationship between vascular permeability and treatment efficacy [115]. This work highlights the potential impact of rigorously studying multimodal therapies (i.e. ChT, ablation, HT) in combination with assessing physiological response to treatment.

3.1. Enhanced permeability and retention effect

Vascular permeability is known to play a central role in tumor accumulation of nanomedicines. Since Matsumura and Maeda published their seminal work detailing the accumulation of large particles (i.e. MW 12,000 – 160,000) within solid tumors [116], the enhanced permeability and retention (EPR) effect has become a central tenant of nanomedicine drug delivery [117]. Vascular permeability is central to this effect whereby long-circulating nanomedicines slowly accumulate at the tumor site by passing through fenestrations in tumor vasculature (i.e. paracellular transport). Retention of nanomedicines at the tumor site is driven by impairment of the lymphatic drainage system. In preclinical models, the EPR effect often produces high levels of tumor accumulation of nanomedicines (i.e. ~0.7 % of injected dose) [118] compared to administration of free drug [119]. For example, in murine 4T1 breast tumors, Laginha et al. demonstrated that Doxil increases the maximum concentration of doxorubicin by 3.6-fold in comparison to free drug [120]. The liposomes greatly increased tumor residence time and increased tumor AUC0-7 days by 87-fold. The EPR effect is known to be quite variable in human subjects, even for the same subtype of cancer [121]. Therefore, it is desirable to increase the potency of the EPR effect by increasing vascular permeability.

3.2. Effect of HT on vascular permeability

Several groups have demonstrated that tumor blood vessel dilation increases paracellular vascular permeability and therefore accumulation of nanomedicines. Seki et al. increased tumor blood flow in mice by topically applying the vasodilator nitroglycerin [122]. Vasodilation doubled the accumulation of 180 nm macromolecular aggregates of polyethylene glycol (PEG)-protoporphyrin within the tumor and resulted in a significant improvement in efficacy. Chen et al. developed a nanomedicine vasodilator by loading the hypertension drug hydralazine into liposomes [123]. Daily injections of liposome encapsulated hydralazine for three days resulted in an increase in vessel diameter as measured by histology and an increase in vascular permeability as measured by injection of fluorescent liposomes. Wei et al. prepared polymeric micelles (100 nm in diameter) that contained both doxorubicin and Cu ions that stimulated endogenous production of NO and dilated tumor blood vessels [124]. Administration of the micelles resulted in threefold greater tumor drug concentrations at 24 h in comparison to equivalent systems that did not generate NO. A significant improvement in efficacy was observed in mice bearing murine 4T1 breast carcinomas. As previously discussed, HT is a safe and effective method to increase tumor blood flow by vasodilation. It is accepted that an important functional effect of HT-induced vasodilation is increased paracellular vascular permeability [125,126]. *In*

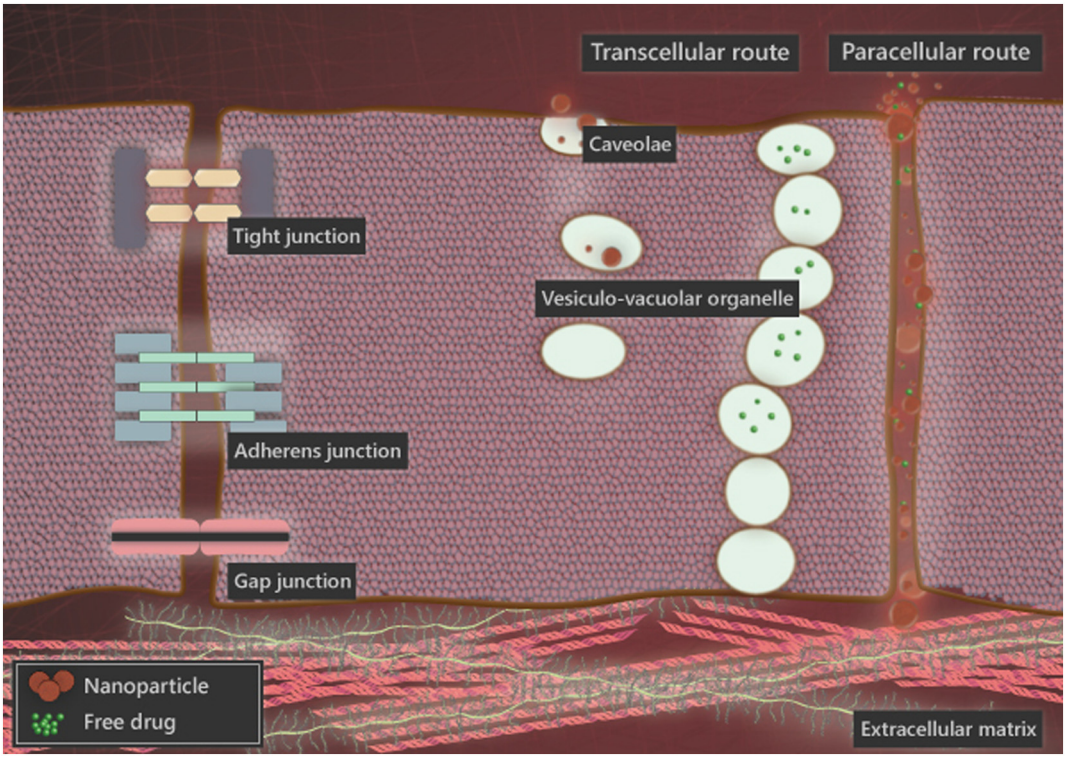

Fig. 3. Transport mechanisms for small molecule and macromolecular agents from the vasculature into the tumor interstitium. Small molecules are transported mainly via vesicular-vacuolar organelles (VVO) whereas larger molecules and nanomedicines are generally thought to enter via paracellular transport. Extracellular matrix (ECM). Adapted under a Creative Commons license (CC BY 3.0) from Azzi et al. [97].

vitro work has shown an increase in gap size between endothelial cells following HT [127]. HT has been shown to result in increased levels of NO within tumors [77]. NO is known to increase vascular permeability [128,129] by controlling junctions connecting vascular endothelial cells (i.e. AJ, TJ) [130].

The potential for increased nanomedicine delivery to tumors mediated by HT-induced increases in vascular permeability has been well characterized. Kong et al. studied the nanomedicine size dependence of HT-mediated vascular permeability [131]. Three different sizes of liposomes (i.e. 100, 200, 400 nm) were produced and accumulation in the tumor interstitium was measured using window chamber models of SKOV-3 human ovarian carcinoma in mice. Preheated tumors were additionally heated for 60 min at 34 or 42 °C following liposome administration. Significantly greater liposome extravasation was observed at 42 °C for all formulations with 100 nm liposomes reaching the highest concentration in the tumor interstitium. There was no measurable extravasation for any of the formulations at 34 °C. Kirui et al. employed plasmon-resonant gold nanorods and low photon flux laser photothermal therapy to induce local HT treatment (i.e. 42 °C, 20 min) [132]. A large, 11-fold increase in tumor blood flow during the course of HT was measured by laser Doppler flowmetry. Vascular permeability was measured by both an albumin-binding small molecule (Evans blue dye; 961 Da) and two separate fluorescent macromolecules (dextrans; 70 kDa and 2 MDa) administered following HT treatment. Tumor extravasation was determined by perfusing the blood volume with saline and measuring Evans blue or dextran concentration in resected tumor tissues. Evans blue dye concentrations in heated tumors were elevated by 2-fold at 1 h and 2.5-fold at 3 h following HT compared to unheated tumors. Concentrations of 70 kDa dextrans (~23 nm in diameter) were ~50% higher in heated tumors compared to unheated from 0 – 24 h following HT. The kinetics of the larger 2 MDa (~56 nm in diameter) dextrans was much different with no difference in accumulation in heated compared to unheated tumors at 1 h following HT. However, at 5, 12, and 24 h following HT the difference in accumulation in heated compared to unheated tumors continued to increase up to ~2.5-fold. The use of three different agents to characterize vascular permeability demonstrates the complex physiological response of tumors to HT and the need for advanced, functional determination of vascular permeability. Several studies have confirmed the ability of HT to increase tumor accumulation of monoclonal antibodies [133–135]. Hauck et al. demonstrated that HT (41.8 °C, 4 h) was able to increase the tumor accumulation of antibodies in human gliomas implanted subcutaneously in mice by 3.5-fold compared to unheated tumors [136]. Interestingly, they found that increased accumulation was not related to changes in tumor IFP and hypothesized that changes in tumor blood flow and vascular permeability are likely to be the main contributors [137].

3.3. Effect of RT on vascular permeability

Similar to blood flow, vascular permeability does not exert a direct effect on RT, but rather mediates therapeutic efficacy through pO_2, pH, and IFP. However, RT can exert important vascular effects. Song and Levitt measured vascular permeability in rats bearing subcutaneous Walker 256 breast carcinomas using radiolabeled albumin [138]. They observed increased vascular permeability in this tumor model following single fraction RT ranging from 2 – 20 Gy [45]. Single fractions of 2.5, 5, and 20 Gy all resulted in increased vascular permeability after 24 h [139]. This was interesting because 20 Gy resulted in decreased blood flow at this time point, whereas 2.5 Gy increased blood flow, 5 Gy had no impact. After 48 h the single 20 Gy fraction substantially reduced vascular permeability whereas it took 8 – 9 days to observe a drop in vascular permeability in the other treatment groups. A similar phenomenon was observed with regards to the impact of RT on the delivery of therapeutics. In intramuscular ovarian (OCa-1) tumors in mice, Li et al. reported that a 15 Gy RT treatment increased serum VEGF concentrations and increased vascular permeability as measured by albumin-bound Evans blue dye [140]. This resulted in increased tumor accumulation of a water-soluble polyglutamic acid-paclitaxel conjugate and increased therapeutic efficacy relative to either ChT or RT alone.

Davies et al. studied the effect of single dose and fractionated RT on Doxil® efficacy in human osteosarcoma xenografts [141]. They found that both single (8 Gy) and fractionated (3 x 3.6 Gy) RT decreased vascular permeability as measured by DCE-MRI using low molecular weight contrast agents. However, RT increased Doxil accumulation ~2- to 4-fold. The authors note the discrepancy in molecular weight and transport kinetics between their therapeutic and vascular permeability imaging agent. It is presumed that RT disrupts the vasculature, altering permeability, while at the same time affecting tumor IFP. Separating the contribution of these two impacts on the accumulation of nanomedicines is challenging and will be further discussed in Section 6.

HT can increase vascular permeability, increasing the accumulation of ChT.

4. Tumor pO_2

Tumor pO_2 plays an important role in dictating cancer biology and response to a range of therapies. While, tumor physiology (e.g. perfusion of blood within the tumor) plays a central role in dictating pO_2. Hypoxia is defined as a reduction in pO_2 levels within tissues that results in impairment of normal biological function [142]. The related condition, hypoxemia, occurs when there is an O_2 deficit in arterial blood [143]. Several major factors contribute to the onset of hypoxia in tumors [144]. It is well understood that the rapid formation of the vascular network within tumors often results in distances between capillaries that are longer than the diffusion distance of O_2 (i.e. ~100 –200 μm) [145,146]. This is referred to as diffusion-limited or chronic hypoxia as the underlying biological conditions will continue to persist [147]. Conversely, intermittent vascular fluctuations can result in rapid onset hypoxia commonly referred to as perfusion-limited, cycling, or acute hypoxia [148]. Cycling hypoxia constantly occurs throughout the tumor volume and can be driven by several factors [149]. The most important driver of cycling hypoxia is red blood cell flux through microvessels [150]. Kimura et al. employed window chamber models of R3230Ac mammary adenocarcinoma in rats along with fluorescence microscopy and O_2 microneedles to measure perivascular pO_2 [151]. They demonstrated that red blood cell flux correlated with pO_2 and decreases in red blood cell flux were sufficient to induce cycling hypoxia (i.e. tissue $p0_2 < 3$ mmHg). pO_2 correlated more closely with red blood cell flux in regions of lower vascular density. O_2 consumption and vascular stasis can also induce acute hypoxia, although these phenomena are less common. O_2 consumption within the tumor can reach levels such that hypoxemia is induced within the arterial blood supply, causing hypoxic conditions in tumor tissue further downstream [144]. Vascular disrupting agents and RT can result in vascular shutdown leading to acute hypoxia and cancer cell necrosis [152]. The pO_2 in arterial blood is ~75 – 100 mmHg [153], whereas in normal tissue it is frequently between 10 – 80 mmHg, depending on the tissue type [154]. Tumor tissue pO_2 is frequently less than 5 – 10 mmHg and this threshold can be used to designate regions of hypoxia [155]. Dewhirst et al. measured arteriolar and peri-arteriolar pO_2 in tumor and normal tissue in rats bearing R3230Ac carcinomas implanted in window chambers [156]. Arteriolar pO_2 averaged 97 mmHg, while there was a statistically significant difference in peri-arteriolar pO_2 in tumor (i.e. 32 mmHg) compared to normal tissue (i.e. 51 mmHg). For *in vitro* studies, moderate hypoxia is often induced by exposing cells to environments containing ~1% O_2 [154]. These states can be maintained in order to mimic chronic hypoxia. Acute hypoxia can be simulated *in vitro* by cycling exposure of cells to ~0.1% O_2 [154]. For preclinical studies, hypoxia is often induced in animals by supplying breathing gases containing lower concentrations of O_2 (e.g. ~5 – 10%) compared to the 21% O_2 that is commonly found in air [157].

4.1. Biological effects of hypoxia

The effects of hypoxia on tumors have been studied extensively. Indeed, the 2019 Nobel Prize in Physiology or Medicine was awarded to three scientists for their work in elucidating the role of hypoxia inducible factors (HIFs) [158]. HIFs are transcription factors that modulate genes in response to low pO_2 within tissues in order to allow cells to better survive in hypoxic conditions [159]. Many important studies have demonstrated HIFs exert control over: angiogenesis [160], metabolism [161], intravasation [162], metastasis [163], cancer stem cells [164], and immune evasion [165] in tumors [166]. Despite the mechanisms that allow tumor tissue to survive under hypoxic conditions, prolonged hypoxia is known to lead to cell death and areas of necrotic tissue within tumors [142]. Given that hypoxia is implicated in so many disease processes that increase the risk of cancer, it is not surprising that hypoxia is a major risk factor for patients with many types of cancers. Several current clinical trials involve detecting hypoxia for patient stratification and novel, hypoxia-activatable ChT are in development [167]. However, given the influence of pO_2 on the efficacy of a number of therapeutic strategies, measurements of hypoxia (i.e. baseline and post-treatment) should be incorporated into a wider range of clinical trials, particularly those purporting to alter pO_2 [168].

4.2. Hypoxia inhibits the efficacy of RT

Hypoxia research has been prioritized largely because of the impact of pO_2 on the efficacy of RT. Clinically, most RT is administered by ionizing radiation that consists of photons, a form of energy with relatively low linear energy transfer (LET) [169]. Proton and carbon beam RT are higher LET modalities, exert more direct damage to target DNA, and therapeutic effect is less dependent on pO_2 [170–172]. Conversely, most DNA damage caused by standard photon RT (referred to throughout this text as RT) is the result of energy absorption within tissue creating free electrons and subsequently free radicals that damage DNA [173]. The oxygen fixation hypothesis states that in the presence of O_2, damage induced by free radicals is made permanent [174]. Many of the free radicals produced are highly reactive, oxygen containing molecules known as reactive oxygen species (ROS) [175]. In hypoxic environments, fewer ROS are produced when cells are irradiated [176]. Many studies have shown a dependence of RT efficacy on pO_2 both *in vitro* [177–179] and *in vivo* [180–182]. The O_2 enhancement ratio (i.e. the factor by which the presence of O_2 increases the cell killing of RT at an isoeffect) is generally reported to be ~2 – 3 [183]. Regions of acute hypoxia that contain less than 5 mmHg pO_2 tend to be highly radioresistant [183]. Given the detrimental effect of hypoxia on RT, there is great interest in identifying patients with hypoxic tumors and developing approaches to sensitize hypoxic tumor regions to RT.

4.3. Treatment options for hypoxic tumors

Traditional ChT and RT approaches are less effective in treating cancer cells within hypoxic environments compared to cells under normoxic conditions [184,185]. Limited efficacy of conventional approaches is of concern given the dangerous potential for invasive growth and metastatic spread [166]. The two main therapeutic approaches to treating hypoxic tumors involve: (1) novel classes of drugs that are active under hypoxic conditions and (2) changing the oxygenation conditions within the tumor.

4.3.1. Direct treatment of hypoxic tissue

The first challenge that needs to be overcome in ChT treatment of hypoxic cancer cells is delivering the drug to the affected area. Hypoxic tumor regions lack functional vasculature, preventing access for many traditional ChT drugs that are only able to diffuse short distances from blood vessels [186]. Diffusion is physically limited by stromal components of the tumor microenvironment, and also by consumption of ChT by cancer

cells [186]. Lack of nearby functional vasculature is an even bigger impediment for nanomedicines because they rely on convection from blood vessels for access to cancer cells. Small molecule agents that are able to access hypoxic tumor regions are further hindered by the biological differences between hypoxic and normoxic cancer cells. Hypoxic cancer cells divide less rapidly, limiting a primary mechanism of action of many ChT [187,188]. In order to overcome these challenges, hypoxia-activated prodrugs that undergo chemical reduction in regions with low pO_2 have been designed [189]. However, to date clinical success has been lacking. Spiegelberg et al. attribute this to clinical trial designs that do not incorporate hypoxia biomarkers and lack corresponding patient stratification [190]. While RT is known to be less effective in treating hypoxic cancer cells, a number of radiosensitizers have been developed in order to overcome this issue [191]. Most radiosensitizers designed to act in hypoxic cells replace oxygen in forming reactive free radicals that can interact with and damage DNA following RT [192]. Clinical development of these agents has been stunted by an inability to balance the toxicity of the agents and the high doses required for radiosensitization [193]. Clinical progress has also been hampered by a failure to select patient populations with hypoxic regions within tumors [191].

Many researchers consider the treatment of hypoxic tumors to be the most compelling use of HT [61,184]. A great deal of research has been done on the ability of HT to treat hypoxic cells [194–196], but rather than directly affecting hypoxic cells, HT exerts several indirect effects that impact hypoxic tumors. HT is able to directly kill cells given sufficient time and temperature exposure, although the cytotoxic threshold varies depending on cell type [197]. The HT-induced cell killing process is not perfectly understood, but combines apoptosis, necrosis, and mitotic catastrophe [198,199]. It is believed that protein denaturation is the cause of these cytotoxic effects [61]. HT is able to exert a greater cell killing effect in hypoxic cell populations, but rather than being driven by pO_2 within the cells, the main factors seem to be pH and possibly nutrient deprivation [200,201]. The studies by Overgaard and Bichel as well as Gerweck et al. noted that the increased sensitivity to HT observed under hypoxic conditions can be reversed by returning the pH to physiological conditions. Furthermore, chronic hypoxia conferred much more sensitivity to HT compared to acute hypoxia, indicating that nutrient status and the health of cells was more of a determining factor than pO_2 levels. Section 5 describes the processes under which hypoxic tissue often becomes acidic. Cancer cells within hypoxic tumors are often nutrient deprived and exist within a lower pH environment [202,203]. Furthermore, many studies have shown that HT-induced cell killing is increased through combination therapy with agents that limit blood flow and thus decrease tumor pO_2 [204]. While ablative therapies have proven clinical benefit at elevated temperatures (i.e. > 60 °C) [205], clinical translation of direct killing of cancer cells by HT (i.e. ~45 °C) has proven less efficacious [69]. This is largely due to the fact that elevated temperature HT does not act synergistically with established treatment modalities such as RT and ChT. Elevated temperature HT is not as amenable to fractioned treatments suitable for combining with RT and ChT and does not induce the physiological responses discussed in this review that improve RT and ChT efficacy.

Increasing the sensitivity of hypoxic cells to RT is a more promising clinical approach and is important given the radioresistance normally associated with hypoxic cells. HT sensitizes hypoxic cells to RT both by inhibiting DNA damage repair induced by RT and by increasing tumor pO_2 therefore increasing DNA damage. HT sensitizes all cells to RT in a time dependent manner by degrading proteins related to DNA damage repair [206,207]. Repeated studies have shown this sensitization to have the biggest impact when RT and HT are delivered simultaneously with decreasing sensitization as HT is delivered at longer time intervals before or after RT [23,208]. This method of RT sensitization affects both hypoxic and normoxic cells equally because the mechanism of sensitization is not O_2-dependent, but related to DNA damage repair [209]. However, HT is able to preferentially sensitize hypoxic cells to RT *in vivo* by increasing tumor blood flow and therefore tumor pO_2. Iwata et al. demonstrated that several different HT protocols can increase tumor pO_2 in both mice and rats [210]. HT (41.5 °C, 1 h) increased pO2 from 6.5 to 16.6 mmHg. This effect was persistent with increased pO_2 still measured 24h following HT. A similar effect was noted in rat tumors with pO_2 increasing from 3.7 to 12.2 mmHg following HT (42.5 °C, 30min). Many preclinical studies have demonstrated that adding HT to RT regimens improves the treatment of hypoxic tumors [211–213]. Sun et al. examined the effect of HT on hypoxia in HT29 human colorectal adenocarcinomas [93]. Tumors were heated for 45 min at 41 °C and hypoxia was assessed prior to HT using pimonidazole and during or after HT using EF5. Perfusion was assessed by intravenous Hoechst 33342 assessment prior to sacrifice and CD31 immunohistochemistry. This HT protocol increased perfusion and reduced overall hypoxia. However, the decrease in hypoxia was not sustained and did not persist 24 h following HT. Furthermore, in addition to a reduction in the overall hypoxic fraction of the tumor, new regions of hypoxia were also reported following HT. However, it is possible that these were normal temporal changes in tumor pO_2 unrelated to HT treatment. Griffin et al. employed water bath induced HT at 41.5 °C for 45 min to study the effect of radiosensitization in fibrosarcoma and mammary carcinoma models in mice [214]. They found that HT decreased the fraction of hypoxic cells in both tumor models by ~3.5-fold. In both tumor models, HT significantly improved the efficacy of a single 20 Gy RT fraction in terms of tumor growth delay and surviving cell fraction as determined by *ex vivo* clonogenic assay.

4.3.2. Increasing tumor pO_2

As discussed earlier in Section 2 of this review, HT can increase tumor blood flow. Sun et al. demonstrated that HT (41 °C, 45 min) -mediated increases in blood flow resulted in increased tumor pO_2, but the impact was short-lived [93]. In canine tumors of up to 400 cm^3 in volume, Thrall et al. studied the effect of two thermal doses of MW HT on tumor pO_2 [215]. Dogs received a total dose of either 10 or 40 CEM43*T90* (isoeffective cumulative exposure that considers the 90th percentile temperature as opposed to the median temperature for CEM43). Both thermal doses were able to increase oxygenation in tumors exhibiting low pO_2 (0 – 4.1 mmHg) prior to treatment. However, the higher thermal dose reduced pO_2 in tumors with higher initial oxygenation values. Thermal dose of HT and baseline levels of pO_2 must be carefully considered in treatment planning. Thrall et al. built on this study by examining the effect of fractionating treatments with equivalent total thermal doses of ~30 CEM43*T90* in 37 spontaneous canine tumors with dogs receiving 1 vs 3 – 4 fractions per week [216]. While there was no significant difference in oxygenation effect between the treatment groups, a significant increase in tumor pO_2 compared to baseline was recorded for both treatment groups. This study is significant in that it demonstrates the ability of repeated HT treatments to increase tumor pO_2 over a significant period of time (i.e. 5 weeks). To date, optimized fractionation of thermal dose has yet to be determined in large animal models and human subjects.

Other approaches to increase tumor pO_2 have focused on temporarily increasing blood pO_2 and include hyperbaric O_2 chambers [193,217], administration of 100% O_2 or carbogen (5% CO_2, 95% O_2) [183], artificial O_2 carriers based on either hemoglobin or perfluorocarbon emulsions [218] [191], and drugs such as efaproxiral that modulate O_2-haemoglobin binding affinity [219,220]. These interventions are transient whereas RT is frequently fractionated over many days and ChT can act over a longer timescale, making a more sustained increase in tumor pO_2 desirable. The excellent safety profile of HT makes it a more compelling candidate compared to some other interventions. However, the most efficient method to increase tumor pO_2 is to reduce O_2 consumption within tumors [221]. Zanella et al. demonstrated that metformin was able to decrease O_2 consumption of several cancer cell lines *in vitro* and that this translated to a decrease in hypoxia within tumors following intravenous administration (100 mg/kg) of metformin [222]. They further demonstrated that this decrease in pO_2 led to improved RT (15 Gy single dose) efficacy in tumor models in mice bearing

HCT116 human colorectal carcinomas. The promise of clinical translation was suggested by a retrospective study of 504 patients that had received RT. Patients receiving metformin exhibited a significant decrease in early biochemical relapse. In another study, Benej et al. employed a clinically approved drug in order to reduce O_2 consumption and increase tumor pO_2 [223]. Papaverine reduced hypoxia and significantly increased RT (5 Gy single fraction) efficacy in EO771 murine breast tumors and A549 human lung tumors in mice.

Tumor vascularization was identified by Judah Folkman as a critical aspect of tumor development [224]. Hanahan and Folkman termed the rapid onset of neovascularization and ensuing tumor growth the 'angiogenic switch' [225]. The signaling protein VEGF, specifically the type VEGF-A, is largely responsible for regulating this process [226–228]. Following this angiogenic switch, many tumors exhibit tortuous, heterogeneous vascular structures that deliver insufficient O_2 and nutrients, while also preventing sufficient delivery of ChT. Sustained angiogenesis was included as a hallmark of cancer by Hanahan and Weinberg in their seminal paper published in 2000 [229]. Rakesh Jain has proposed the concept of vascular normalization whereby the tumor vasculature is modified to more closely resemble the vascular networks of normal tissue [230]. This is achieved by correcting the imbalance between pro- and anti-angiogenic signaling via administration of a carefully determined regimen of anti-angiogenic therapy [231]. However, clinical translation of this therapeutic intervention is difficult because the dose of anti-angiogenic drugs must fall within a narrow window such that the dose is sufficient to stem unchecked growth, but not so high as to shut down vasculature growth completely [232]. Patient heterogeneity and spatial and temporal variability in tumor vascularization and angiogenic signaling further complicate dose selection and treatment. While it may seem counterintuitive, low dose anti-angiogenic therapy has led to increased tumor pO_2 in multiple preclinical models [233–235].

HT has the potential to play a similar role in rebalancing pro- and anti-angiogenic factors, resulting in more normal tumor vasculature. As previously discussed, application of HT (i.e. < 42 °C) can increase blood flow and pO_2 within tumors. An increase in pO_2 results in a reduction of HIFs and a corresponding decrease in pro-angiogenic signaling. Conversely, HT at higher temperatures (i.e. > 42 °C) can damage the vasculature and result in an increase in pro-angiogenic factors. Kanamori et al. applied water bath HT for 30 min at 44 °C to mice bearing squamous cell carcinomas (SCC VII) [236]. Under these conditions HT can have anti-vascular effects. Indeed, HT or intravenous administration of the anti-angiogenic drug TNP-470 both resulted in necrosis within the central regions of tumors and an increase in VEGF expression. However, Sawaji et al. found a significant decrease in serum levels of VEGF in patients 2 – 3 weeks following completion of whole body HT treatments (42 °C, 1 h) [237]. Time-dependent HIF and VEGF expression may further complicate pursuing HT as a strategy for balancing angiogenic factors. Moon et al. determined that at temperatures between 41-44 °C, HT induced HIF activation *in vitro* in two human cancer cell lines [238]. They further measured increased HIF and VEGF expression *in vivo* up to 24 h and 48 h, respectively, following treatment. The temperatures employed in these studies are likely also critical in achieving the desired therapeutic outcomes. It is possible that a version of vascular normalization can be achieved through the application of HT rather than pro- and anti-angiogenic factors. Interestingly, Sen et al. reported that the use of low temperature, whole body HT (39.5 °C, 6 h) was able to increase blood flow and decrease the hypoxic fraction of the tumor as measured by intravenous administration of pimonidazole hydrochloride and immunohistochemistry analysis [239]. In addition to increasing blood flow and reducing hypoxia, HT reduced IFP and improved the efficacy of RT. It is presumed that ChT efficacy would also be enhanced.

HT can increase pO_2, improving RT efficacy.

5. Tumor pH

5.1. Altered glucose metabolism

The acidic or basic nature of the tumor microenvironment is of interest from a therapeutic standpoint and it is inextricably linked to tumor pO_2. The tumor microenvironment tends to be more acidic compared to normal tissues and this is largely caused by altered glucose metabolism. Cells within the body derive energy from cellular respiration, whereby glucose and oxygen are converted to carbon dioxide and water via an intermediary pathway involving the breakdown of pyruvate via the citric acid cycle and oxidative phosphorylation [240]. However, the tumor microenvironment is often deficient in oxygen as previously discussed in Section 4. The Nobel laureate Otto Warburg hypothesized that in the absence of oxygen, cancer cells produce energy via anaerobic respiration [241]. The anaerobic respiration pathway begins in the same manner as aerobic respiration, but ends with the conversion of pyruvate to lactate, a process that produces far less energy. Adenosine triphosphate (ATP) molecules are the standard energy units of molecular biology and only two are produced when converting glucose to lactate. Conversely, full aerobic respiration can result in production of far more ATP molecules (i.e. ~30 – 38 total) [240]. However, the rate at which anaerobic glycolysis occurs is much faster, allowing the two forms of metabolism to produce similar amounts of ATP over the same timeframe [242]. Therefore, cells producing ATP via conversion of glucose to lactate consume far greater amounts of glucose compared to normal cells under standard aerobic respiration. It has been observed that even in the presence of oxygen, many cancer cells metabolize glucose to lactate rather than the oxidative phosphorylation pathway that produces more ATP. In the 2011 update to their Hallmarks of Cancer, Hanahan and Weinburg added 'Reprogramming Energy Metabolism' as an emerging hallmark, specifically recognizing the work of Warburg from 80 years prior [243]. This approach results in tumors consuming far more glucose than normal tissue. Indeed, this increased glucose consumption is the basis on which fluorodeoxyglucose-PET imaging differentiates between cancerous and normal tissue [244]. While there are similarities between tumor regions with low pO_2 and low pH, it is important to note that these regions do not necessarily co-localize [245]. Variability in intravascular blood pO_2 and blood flow can cause spatial variations in both pO_2 and pH distribution within the tumor, contributing to this disparity.

While there is ongoing debate as to the advantage gained by cancer cells through this metabolic reprogramming, the effect on the pH of the tumor microenvironment is clear [242]. Intracellular conversion of glucose to lactate (i.e. the conjugate base of lactic acid) produces hydrogen ions within the cytosol [246]. While lactate can be used as a nutrient by cancer cells [247,248], the hydrogen ions are a less desirable product. The intracellular pH (pH_i) of cancer cells is generally slightly more alkaline (i.e. ~7.3–7.6) than that of normal cells (i.e. ~7.2) [249–251]. In order to maintain these pH_i conditions, cancer cells shuttle hydrogen ions across the cell membrane primarily via the sodium/hydrogen ion exchanger NHE1 and the hydrogen/lactate transporter [252], resulting in acidification of the surrounding tumor microenvironment. Gillies et al. surveyed the literature and found that extracellular pH (pH_e) was generally more acidic than pH_i in a wide range of preclinical cancer models [253]. Prescott et al. first demonstrated that this was also the case in spontaneous tumors in a study of 31 dogs with soft tissue sarcomas [254]. Despite this overall trend holding, they observed wide variability in both pH values and cases where $pH_e > pH_i$. Overall, altered glucose metabolism results in pH_e of the tumor microenvironment (i.e. ~6.8–7.0) being more acidic compared to pH_e of many normal tissues (i.e. ~7.4) [251].

5.2. Biological impact of acidic tumor pH

The pH of the tumor microenvironment is particularly important because multiple studies have correlated acidic conditions within the tumor with more aggressive disease phenotypes. A large body of research generated by Drs. Gillies and Gatenby has demonstrated that acidic metabolites cleared from tumor tissue can accumulate in surrounding normal tissue, acidifying the tissue microenvironment [255–258]. Normal cells in the surrounding tissue do not effectively maintain transmembrane pH gradients in acidic environments, resulting in lower pH_i and finally cell death. The resulting lack of viable cells and lower pH in the tissue margins bordering the tumor create an ideal environment for the neighboring cancer cells to invade. Metastatic spread is thought to depend on cancers cells undergoing the process of epithelial-mesenchymal transition (EMT) [259]. New research suggests that cells in lower pH environments are more prone to undergoing EMT [260]. Acidic tumor microenvironments are therefore more likely to produce metastatic disease. Low pH tumor microenvironments have also been associated with impairment of immune cell function [261]. Immunosuppression, often mediated by release of immunoregulatory cytokines, allows tumors to grow unchecked by the body's natural defenses [262]. It is therefore of primary concern to effectively treat cancer cells that reside within acidic tumor regions as they are likely to be of a more aggressive phenotype.

5.3. Acidic pH_e compromises therapeutic efficacy

The acidity of the tumor microenvironment has a direct impact on the efficacy of conventional anti-cancer treatments. Many commonly used anti-cancer drugs (e.g. paclitaxel, doxorubicin, mitoxantrone) are less effective when the pH_e is 6.5 rather than 7.4 [263,264]. A major contributing factor to this effect is that many ChT agents are weak bases that are charged in acidic environments, which prevents them from readily crossing the cell membrane [265]. A strategy to avoid this pitfall is to develop ChT drugs that are weak acids that are uncharged in the slightly acidic tumor microenvironment, but charged at more alkaline pH_i. Drugs with these chemical properties would readily cross the cell membrane at tumor pH_e and subsequently become entrapped in cancer cells at pH_i. Gerweck et al. used intravenous glucose administration to slightly acidify the tumor microenvironment and observed an improvement in the anti-tumor efficacy of the weak acid chlorambucil [266]. Conversely, glucose-mediated acidification of the tumor microenvironment had a negative effect on the efficacy of the weak base doxorubicin. This study is an interesting application of leveraging the altered glucose metabolism of cancer cells to tailor the tumor microenvironment and optimize the efficacy of specific drugs. Other work suggests that acidic pH_e can increase the intracellular activity of P-glycoprotein (P-gp) in some cancer cells, resulting in a decrease in ChT cytotoxicity that can be reversed by co-administration of a P-gp inhibitor [267]. P-gp is also known as multidrug resistance protein 1 and plays an integral role in efflux of many traditional ChT from cancer cells, rendering drugs less effective [268]. This effect further hinders the efficacy of many anti-cancer drugs being used to treat cancer cells in acidic microenvironments. The efficacy of several other therapeutic interventions is also compromised at pH_e acidic. While not a dominant effect, particularly compared to the radioresistance of hypoxic cells, studies have also shown that cancer cells exhibit a limited amount of additional radioresistance at lower pH [269,270]. Even newer immunotherapies have been shown to be less effective in treating tumors with acidic microenvironments because of the previously described immune suppression [271]. Thus, it is incumbent to develop alternative treatment strategies that are either efficacious in killing cancer cells at acidic pH or that alter the pH of the tumor microenvironment, making cancer cells more susceptible to traditional ChT.

5.4. HT is preferentially cytotoxic to low pH_i cancer cells

Many studies have demonstrated that HT is much more toxic to cells in acidic environments compared to those at physiological pH [272]. This fact led to the exciting premise that HT could be used to selectively target cancers cells as they often reside in a more acidic milieu compared to normal cells. However, *in vitro* studies blocking the Na+/H+ exchange demonstrated that acidic intracellular, rather than extracellular, pH dictates the sensitivity of cancer cells to HT [273]. It was later demonstrated that blocking the heat shock protein response at acidic intracellular conditions confers heat sensitivity [274]. The dependence of hyperthermic cell killing on acidic pH_i is crucial as it has also been shown that cells chronically exposed to lower pH environments are able to maintain pH_i much closer to physiological conditions [275]. Thus, it would seem that the selective toxicity of HT to cancer cells compared to normal cells is largely limited by the ability of cancer cells to adapt to their microenvironment. However, under conditions of acute hypoxia, cancer cells at low pH_e become unable to maintain a transmembrane pH gradient, resulting in a drop in pH_i [276] that makes these cells sensitive to HT treatment. Spees et al. have completed a thorough study inducing acutely lower pH_i in murine tumors by either glycolytic or non-glycolytic methods [277]. Using ^{31}P-MR spectroscopy (MRS), they determined that both approaches decreased pH_i within the tumor and that for both methods of altering pH_i, there was a linear relationship between pH_i and sensitivity to HT (i.e. lower pHi, more sensitive to HT) (43 °C, 30 min). HT may therefore be an effective method of treating cancer cells under conditions of acidic pH_e and acute hypoxia that would otherwise be RT- and ChT-resistant.

5.5. Raising tumor pH_e to increase ChT efficacy

One treatment approach for acidic tumors is to use therapies that are effective at lower pH. The other approach is to increase the pH_e of the tumor in order to make conventional therapies more effective. One promising approach is being explored by Dr. Gillies group involves oral administration of bicarbonate provided to mice ad libitum in their drinking water [278]. Robey et al. demonstrated that this approach increased pH_e, but not pH_i within tumors and observed a corresponding decrease in metastatic spread of MDA-MB-231 human breast cancer cells [279]. Subsequent work by Pilon-Thomas et al. further demonstrated that oral administration of bicarbonate improved tumor response to several immunotherapies including PD-1, CTLA-4, and adoptive cell therapy [271]. Low pH_e is caused by hydrogen ions produced during glycolysis that are not cleared due to inadequate blood flow and lymphatic drainage in some tumor regions. Therefore, interventions such as HT that increase tumor blood flow could also be effective in raising tumor pH_e. However, most literature does not support the ability of HT to raise the pH of acidic tumor microenvironment. Wike-Hooley et al. summarized a substantial quantity of literature describing the relationship between HT and tumor pH [269]. Citing a number of studies, they concluded that when HT was administered at temperatures above 42 °C, a decrease in the pH of the tumor microenvironment was observed [54,280,281]. They speculate that this may be caused by a reduction in blood flow, in general agreement with current literature indicating that higher HT temperatures (i.e. > ~42 °C) can reduce blood flow in many tumor models. However, the observed reduction in pH could also be the result of a switch towards more anaerobic metabolism. It has been noted that HT can upregulate HIFs [216,238] and that respiration is more sensitive to HT compared to glycolysis [282]. This suggests that an increase in blood flow may be able to raise the pH_e of the tumor closer to physiological levels, albeit in a transient manner, depending on the duration of HT-induced increased in blood flow. Indeed, Jayasundar et al. measured pH_i and pH_e using ^{31}P-MRS and a fiberoptic pH meter during and after 30 min HT treatments at 42 or 45 °C [283]. Treatment at 45 °C resulted in statistically significant decreases in both pH_e and pH_i, whereas 42 °C HT increased pH_e. It is hypothesized

that these pH differences are driven by modulation of blood and could be therapeutically relevant. It is presumed that in many cases lower HT temperatures (i.e. < ~42 °C) are able to increase pH_e within the tumor and improve the efficacy of ChT. In a clinically relevant study examining the effect of tumor pH on the efficacy of RT + HT in spontaneous canine tumors, Lora-Michiels et al. determined that pretreatment pHe is a strong prognostic factor for metastasis-free survival [284]. Of the 30 animals studied, those with tumor pHe >7 had a hazard ratio of 0.29 compared to tumors with a more acidic pH_e. Interestingly, tumors for which pHe reduced following HT had a better prognosis compared to tumors for which pH_e became more alkaline. The preceding discussion on the impact of HT on pH_e indicates a complex relationship with outcomes very susceptible to differences in exposure. While more work is required in this area, it is hoped that the development of MRS capable of non-invasively determining intratumoral pH_e and pH_i will enable elucidation of the relationship between HT and tumor pH.

HT can increase extracellular pH, improving RT efficacy.

6. Tumor IFP

The interstitium is the fluid filled compartment of extracellular matrix that fills the volume between the vascular and lymphatic systems and the parenchymal cells of tissues [285]. The tumor interstitium generally consists of stromal cells such as fibroblasts and immune cells and the interstitial fluid that surrounds them [286]. The tumor interstitium is an important consideration in drug delivery because densely packed stromal cells represent a transport barrier that can be difficult to overcome [287]. This topic has been expertly reviewed elsewhere [35], and largely falls outside the scope of this review, as there have been few studies on the effect of HT on tumor stroma [288]. The exception to this would be the large body of literature studying the impact of elevated temperatures on the immune response. This important topic has been reviewed extensively [2,289–291] and is discussed in the context of drug delivery elsewhere in this special issue. The fluid pressure within the interstitial space impacts drug delivery and can be modulated by various interventions including HT. In normal tissues both the vascular and lymphatic networks regulate fluid pressure within interstitial space [292]. However, in the tumor interstitium, fluid accumulates in a process related to the EPR effect. Fluid collects in the tumor interstitium due to the leakiness of the vasculature and is not adequately removed due to impaired lymphatic drainage [293]. Hydrostatic pressure in capillaries is generally about 10 – 30 mmHg, with pressure dropping along the length of the capillary, resulting in fluid extravasation at the arteriolar end and reabsorption of fluid at the veniolar end [286]. In normal tissue, pressure gradients between the vasculature and interstitium are typically 10 – 40 mmHg [36], resulting in IFP that is often atmospheric (i.e. 0 mmHg) or slightly negative. Leaky vasculature and impaired lymphatic conditions in the tumor interstitium described above can result in the tumor IFP equilibrating with the vascular hydrostatic pressure (i.e. ~10 – 30 mmHg). Tortuous blood vessels can result in higher intravascular pressure [294], further increasing tumor IFP [295]. Boucher and Jain demonstrated in preclinical models that elevated microcapillary pressure correlated with increased IFP [296]. Tumor IFP has typically been measured at 10 – 40 mmHg [295,297–299], with values as high as 100 mmHg having been recorded [300]. Tumor IFP has been thoroughly reviewed elsewhere [36,286]. This section focuses on the implications of altered IFP on the efficacy of ChT and RT as well as the potential role of HT in overcoming these challenges.

6.1. How tumor IFP affects cancer

The effects of pressure on cancer progression are not as well understood as other factors such as pO_2 and pH. While tumors have compromised lymphatic networks, they are not lymphatic naïve. It is well understood that cancer cells can be transported out of the tumor via the sentinel lymph node, often as a first step in metastatic spread [301]. In high IFP tumors, more fluid will be drained through the lymph nodes, increasing the chances for spread of the disease via the lymphatic network [302,303]. Furthermore, the Swartz group have demonstrated the autologous chemotaxis phenomenon wherein cells secrete the chemokine CCL21 that convects along fluid pressure gradients towards draining lymph nodes [304]. Similarly, high IFP in the centre of the tumor causes fluid to flow towards the periphery and into surrounding tissue [305,306]. This can drive invasion of cancers cells as well as signalling molecules that prime the surrounding microenvironment for tumor infiltration [307]. High IFP tumors show greater expression of the proliferation marker Ki-67 compared to low IFP tumors [308], indicating a more aggressive phenotype [309,310].

High IFP can also reduce blood flow, leading to regions of low pO_2 and low pH in tumors and ultimately resulting in necrosis [311]. While it has been suggested that high IFP can also directly occlude tumor vessels [311], it is likely that occlusion requires a significant pressure contribution from growth-induced solid stress [312]. Nathan et al. used real-time PCR to demonstrate another mechanism by which IFP affects tumor vascularization [313]. Osteosarcoma cells cultured *in vitro* under elevated pressures (i.e. 20 mmHg) expressed less VEGF-A (i.e. angiogenic factor) and more VEGF-C (i.e. lymphatic factor) compared to cells cultured at atmospheric pressure. These results mirrored immunohistochemistry data from a patient population in which higher IFP tumors correlated with decreased VEGF-A and increased VEGF-C. There are therefore multiple ways in which tumor IFP affects cancer biology.

6.2. IFP has an adverse effect on ChT and RT

6.2.1. Effect of IFP on drug delivery

IFP exerts a strong influence on the efficacy of ChT as it is a major factor affecting delivery of therapeutics to the tumor site as well as drug distribution within the tumor. The pressure gradient between tumor vessels and tumor tissue dictates the amount of fluid convection into the tumor interstitium. While smaller molecules such as traditional ChT drugs (e.g. doxorubicin, paclitaxel, cisplatin) rely on both diffusive and convective transport, larger molecules such as nanomedicines and antibodies are almost solely dependent on convection for transport [33]. When pressure in the interstitial space is high, convection can be completely eliminated, reducing transport of smaller molecules and preventing transport of large molecules. The impact of elevated IFP on drug transport is becoming a more important issue as many new therapeutic agents for oncology have higher molecular weights than those developed in the past.

Many preclinical studies have demonstrated that elevated tumor IFP is a barrier to macromolecular drug delivery. Eikenes et al. evaluated the effect of IFP on the distribution and accumulation of antibodies in mice bearing human osteosarcomas by measuring both microvascular and interstitial pressures using micropipette and wick-in-needle techniques, respectively [314]. The murine monoclonal antibody TP-3 binds to a specific osteosarcoma-associated cell surface antigen [315] and was administered intravenously to mice. Collagenase administration was found to increase transvascular pressure gradients and decrease IFP, leading to both an increase in antibody accumulation within the tumor and an increase in antibody penetration away from tumor blood vessels. This study underscores the challenge in isolating the individual contribution that each physiological factor exerts on drug delivery. It is likely that degradation of collagen within the extracellular matrix reduces physical barriers to transport and further contributes to interstitial antibody transport. Indeed, it is known that collagen content is inversely related to the apparent diffusion coefficient [316] and thus collagenase administration would increase both convection and diffusion. Netti et al. took the opposite approach and raised vascular blood pressure in order to increase the intratumoral transvascular pressure gradient and thereby increase antibody accumulation within tumors [317]. The effect of both continuous and periodic administration

of angiotensin II were compared in mice subcutaneously implanted with the human colon adenocarcinoma LS174T. The minretumomab antibody binds the TAG-72 antigen present on the cancer cells used in this study. Following radiolabeled minretumomab administration, blood pressure was altered and antibody accumulation in the tumor was measured by gamma camera imaging. Both continuous and periodic administration of angiotensin II were able to increase antibody accumulation in the tumor by approximately 40%. Fan et al. employed a liposome formulation of imatinib in order to reduce tumor IFP in murine B16 melanoma tumors implanted in mice [318]. The response was sustained with statistically significant reductions in tumor IFP at 2.5, 26, and 50 h following liposome administration. Interestingly, the administration of free imatinib did not result in a decrease in tumor IFP. *In vivo* fluorescent imaging demonstrated that tumor priming with imatinib liposomes significantly increased the delivery efficiency of doxorubicin liposomes to tumors. They determined the decrease in tumor IFP resulted from fibroblast inhibition, inhibition of platelet-derived growth factor receptor beta, and the anti-angiogenic effects often associated with imatinib. However, pretreatment with imatinib liposomes increased the efficacy of both doxorubicin liposomes and free doxorubicin to the same degree, indicating that the improvements in efficacy are more complex than solely an increase in drug transport due to convection.

The group led by Rubin at Uppsala University has published extensively on the effect of tumor IFP on drug delivery. The majority of this work has focused on modulating tumor IFP and ChT efficacy through the targeting of platelet-derived growth factor (PDGF) receptor and VEGF receptor. PDGF is known to play a role in blood vessel formation as well as autocrine signalling in epithelial cancers [319]. In 2000 they demonstrated that imatinib, an inhibitor of PDGFR reduced tumor IFP in subcutaneously implanted colon cancers in rats [320]. This resulted in a significant increase in accumulation of ^{51}Cr-EDTA (MW 339 Da) in the interstitial space. Further work showed that imatinib was able to increase the accumulation and efficacy of Taxol® as well [321]. In the studies from the Rubin group Taxol (paclitaxel formulated in 65% buffered saline, 25% ethanol, and 10% Cremophor EL) was administered subcutaneously. Paclitaxel readily binds to protein [322,323]. Therefore, administration via this route presumably results in macromolecular, protein-bound drug reaching the site. Later work administered the VEGF inhibitor bevacizumab to mice bearing subcutaneous human KAT-4 thyroid carcinomas that are known to express high amounts of VEGF [324]. Once again, anti-angiogenic treatment resulted in a sustained decrease in tumor IFP. The combination of imatinib with vatalanib, another VEGF inhibitor, demonstrated that combining PDGF and VEGF inhibition is able to lower tumor IFP to more than either single agent [325]. Despite reducing vascular density in the tumor, while not having an effect on tumor growth, the combination therapy enhanced the efficacy of Taxol more than either imatinib or vatalanib alone. However, the authors found that the sequencing and timing of inhibitor administration was crucial. Conversely, Tailor et al. demonstrated that while the VEGF and PDGF inhibitor pazopanib is able to decrease tumor IFP, there was also an increase in hypoxia, and no significant impact on Doxil accumulation [326]. They speculate that the same vascular normalization that reduced tumor IFP is also responsible for decreasing vascular permeability and nanomedicine accumulation within the tumor. Reducing perfusion in order to reduce tumor IFP and enhance ChT is a delicate proposition that must be closely monitored.

6.2.2. Effect of IFP on RT

There is mounting evidence that tumors with elevated IFP are less responsive to RT [327]. Landmark clinical trial results published by Milosevic, Fyles, and Hill in 2001 revealed that cervical cancer patients with higher IFP had a poorer prognosis following RT treatment [297]. A modified wick-in-needle technique was used to make IFP measurements at multiple positions within each tumor for 77 individual patients. An additional prospective study at the same institution determined that IFP is an independent prognostic factor in cervical cancer patients [328]. Despite once again confirming that high IFP was correlated with poor survival outcomes following RT, there was no correlation between IFP and tumor oxygenation. Furthermore, there was no link between IFP levels and other clinical prognostic factors for cervical cancer (i.e. tumor size and lymph node involvement). Later work confirmed that IFP prior to RT is a prognostic factor for survival of cervical cancer patients [329]. The same correlation between high IFP and radioresistance has also been observed in preclinical models. Rofstad et al. measured IFP using a wick-in-needle technique for intradermal tumors and a Millar SPC 320 cathether for window chamber model xenografts [330]. Using cutoffs of ≤ 7 mmHg and ≥ 9 mmHg, they determined that lower IFP tumors exhibited better tumor control for all treatment doses ranging from 25 – 45 Gy. Importantly, this study also measured a positive correlation between IFP and vessel tortuosity. The mechanism by which high IFP induces radioresistance is not known, but Rofstad et al. speculate that it may result from increased VEGF-A signaling and/or increased tumor cell clonogenicity [331]. Milosevic et al. also speculated that high IFP results in increased angiogenesis, partially negating the anti-vascular effect of RT [330]. Notably, high tumor IFP has been shown to hinder RT treatment efficacy, regardless of pO_2 [331].

6.3. Treatment approaches for high IFP tumors

High tumor IFP is a barrier to effective RT and macromolecular ChT. As a result, there is interest in interventions that can reduce tumor IFP and enhance the efficacy of existing treatments. The following sections review ChT, RT, and HT interventions that have been evaluated to reduce high tumor IFP in order to improve treatment potential.

6.3.1. ChT-based approaches to treating high IFP tumors

Drug-based strategies are not generally developed with the explicit goal of reducing tumor IFP, however several clinical studies have demonstrated that small molecule ChT has the potential to do so. Curti et al. characterized tumor IFP in melanoma lesions in patients receiving ChT [300]. There was a strong correlation between patients who responded to treatment and those who experienced a drop in tumor IFP. Mean tumor IFP doubled to 54 mmHg in non-responders, but dropped to 0 mmHg for patients who responded to treatment. Therefore, ChT can potentially reduce tumor IFP, but this approach leaves non-responding patients with more aggressive, difficult to treat, high IFP tumors. Work by Taghian et al. measured IFP in breast cancer patients receiving sequential treatments of paclitaxel (nine cycles of 80 mg/m^2 qw) and doxorubicin (four cycles of 60 mg/m^2 q2w) [332]. The order of treatment was varied for the two patient groups. Tumor IFP was reduced by 36% in patients receiving an initial paclitaxel treatment, while doxorubicin treatment did not affect IFP. By the end of both treatments, tumor volume was not significantly different in the two treatment groups. Perhaps if the doxorubicin had been encapsulated in a nanomedicine, the treatment order would matter as the decrease in IFP resulting from paclitaxel therapy would likely yield an increase in doxorubicin accumulation. Griffon-Etienne et al. have demonstrated the mechanism whereby conventional ChT reduces IFP in two mouse models [333]. Taxanes caused apoptosis of neoplastic cells within the tumor, reducing solid tissue pressure and decompressing blood vessels. An increase in tumor blood vessel diameter was measured along with a drop in tumor IFP.

Several preclinical, drug-based approaches to reduce tumor IFP have focused on modifying the tumor vasculature. Several studies that employed agents including angiotensin II [317], imatinib [318,320,325], bevacizumab [324], and vatalanib [325] have already been discussed in the context of their utility in increasing the accumulation of macromolecular drugs (Section 6.2.1). Skliarenko et al. examined the ability of the anti-vascular agent ZD6126 to lower tumor IFP in murine fibrosarcoma (KHT-C) and human cervical cancer (CaSki) tumors growing intramuscularly in mice [334]. The intention of this therapy was to disrupt the tumor vasculature and prevent further fluid

accumulation in the interstitial space. Indeed, this was effective with IFP being reduced after 72 h to 25% of the pretreatment values in KHT-C tumors and 30% of the original IFP in CaSki tumors. This intervention would not be appropriate for combination with ChT as vascular transport would be compromised, reducing overall drug delivery. However, there are possible synergies with RT. Indeed, other anti-vascular agents such as combreastatin A4 are known radiosensitizers. It is possible that reduction of IFP is one mechanism of action. Podobnik et al. administered the vasodilating agent hydralazine to mice bearing subcutaneous anaplastic sarcoma F (SaF) tumors [335]. IFP was measured by the wick-in-needle technique and found to drop by a mean amount of 33% at 30 min following hydrazine injection. There are therefore several different means by which drugs can be employed to decrease tumor IFP.

6.3.2. RT-based approaches to treating high IFP tumors

Znati et al. employed the wick-in-needle technique to measure the effect of RT on tumor IFP [336]. Fractionated RT at total doses of 10 and 15, but not 5 Gy reduced tumor IFP 24 h following treatment. Single fractions of 10, 20, and 30 Gy also resulted in a significant, prolonged decrease in IFP. Of note, a single 30 Gy treatment reduced IFP by 35% after 5 d and preceded a significant arrest of tumor growth. Our research team studied the effect of RT on IFP in mice implanted with MDA-MB-231 human breast cancer tumors [337]. Similar to Znati et al. we found that higher doses of RT were required to cause a sustained decrease in IFP. Single fraction RT of 5 and 10 Gy did not result in statistically significant reductions in IFP, while 15 Gy resulted in lower IFP in tumors at 1 and 24 h post-RT. The effect of RT on tumor IFP is multifactorial and includes vascular damage, which reduces fluid flow to the tumor, as well as direct cancer cell killing, which reduces cell density. Clinically, there has been little study of the effect of RT on tumor IFP. Roh et al. measured IFP in seven cervical cancer patients during fractionated RT [338]. RT doses ranged from 39.6 to 60.0 Gy and decreased tumor IFP was observed in four patients while an increase was measured in the other three patients. It is therefore difficult to conclude that RT was able to reduce IFP. However, a reduction in IFP correlated with better clinical outcomes and the authors suggest that IFP response to RT could be a prognostic indicator of response to RT.

6.3.3. HT-based approaches to treating high IFP tumors

There have been several reports that HT is able to reduce tumor IFP, although the mechanism of action has not yet been demonstrated. The first evidence of the ability of HT to reduce tumor IFP came from Leunig et al. in 1992 [339]. This study employed an allograft implantation of hamster melanoma into the dorsal skin and water bath HT was administered at 43 °C for 30 or 60 min. HT treatment under these conditions may reduce blood flow, but blood flow was not measured in this study. At 48 h following HT, tumor IFP was significantly lower in animals that had received HT for 30 min compared to control animals and significantly lower in the 60 min HT group compared to 30 min. Tumor growth delay was also recorded in animals receiving HT compared to control animals, suggesting that thermal effects were not just physiological, but induced vascular damage and/or direct tumor cell killing. Sen et al. employed a temperature controlled chamber to deliver mild, whole body HT for 6 h at 39.5 °C (CEM43 = 2.8 min) to immune competent mice bearing either murine colon tumor 26 (CT26), murine mammary 4T1, or murine melanoma B16.F10 tumors [239]. A wick-in-needle setup with a Millar MikroTip catheter transducer was used to measure tumor IFP. A significant reduction in tumor IFP was measured for all three tumor models following HT and IFP continued to decrease for up to 24 h. Indeed, a sustained response was observed in colon and melanoma tumors with IFP remaining at a reduced pressure 24 h post HT (data was not reported for breast tumors). HT did not affect tumor growth for the colon or melanoma tumor models (data was not reported for breast tumors). Sen et al. also reported an increase in blood flow and blood vessel perfusion in the tumor following HT. It therefore presumed that this lower temperature HT protocol would be beneficial to both small and large molecule ChT and RT. The reported sustained reduction in IFP is particularly important in ensuring sufficient time for macromolecular drugs to accumulate. Our group measured a significant reduction in tumor IFP in MDA-MB-231 breast cancer tumors that were heated for 20 min at 42 °C (CEM43 = 5 min) [337]. Tumor IFP was reduced from 18 to 7 mmHg during the application of HT. This treatment also resulted in a significant increase in both the accumulation and distribution of a nanomedicine contrast agent as well as improving the efficacy of Doxil. While higher temperature HT treatments are able to decrease IFP by disrupting tumor vasculature, lower temperature HT treatments that increase blood flow as well as decrease tumor IFP are a more promising strategy in combination with ChT.

HT can reduce IFP, improving ChT efficacy.

7. Triggered Release from Nanomedicines

Nanomedicine formulations have been designed to release their drug cargo as a result of exposure to many external stimuli including heat, pH, irradiation, specific wavelengths of light, US and several other externally applied and endogenous factors [340–343]. Most of these topics fall outside the scope of this review, but the subject at hand necessitates a brief discussion of heat-triggered drug release, specifically thermosensitive liposome technology. This subject has been thoroughly reviewed elsewhere [344–346], but any discussion of combining HT and drug delivery would be incomplete without discussing recent advancements in thermosensitive drug delivery. Also briefly discussed are RT-triggered and pH-triggered nanomedicines.

7.1. HT-triggered release

The most clinically advanced HT-triggered nanomedicine is Celsion's ThermoDox®, which is currently undergoing Phase III clinical testing in primary liver cancer (NCT02161562). Thermosensitive ChT have been studied extensively and are discussed in other reviews in this issue [347,348]. Advances in the thermosensitive drug delivery platforms have been enabled by engineering developments in the technology used to heat tissue [349,350]. Scientific advances in both HT-application and drug delivery technologies combine to allow for spatially focused, well-controlled drug release within target tissues. Targeted, heat-triggered release is of particular interest in oncology where toxic ChT agents can have debilitating off-target effects in normal tissue. TSL development has been built on decades of research characterizing the temperature-dependent permeability of lipid bilayers [351,352] and liposomal drug carriers [353,354]. Lipid bilayers have a characteristic temperature known as the melting temperature (T_m), above which the lipids exhibit fluidity of movement and below which the bilayer is referred to as solid with comparatively little displacement of the lipids. Liposomes are nanometer- or micrometer-sized vesicles with an outer lamellae consisting of a lipid bilayer. Thermosensitive liposomes exploit this T_m in order to quickly release drug in response to HT.

While the majority of clinically approved nanomedicines are liposomes [355], their primary clinical benefit has been a reduction in ChT induced toxicity. This directly results in improved quality of life for patients, but there is still an unmet need to develop more efficacious nanomedicines. Dr. Gregoriadis led the pioneering development of liposomes as drug delivery carriers in the mid-1970s [356,357]. It was not long after in 1978 that Yatvin et al. reported on the first use of heating liposomes above their T_m in order to trigger release of encapsulated cargo [358]. These liposomes were composed primarily of DPPC, with DSPC added to raise the T_m. A 7:3 DPPC:DSPC liposome formulation combined with microwave heating of tumors to 41.5 – 42.5 °C increased tumor accumulation in mice 3-fold compared to free drug or liposomes administered without HT [22]. However, advancement of this research was limited by existing clinical heating technologies as well as insufficient stability of the liposomes through a lack of steric stabilization

which, as the authors concluded, resulted in aggregation and formation of multilamellar structures. The field of liposome research was greatly advanced in the early 1990s with the discovery that conjugation of PEG polymer chains onto the surface of liposomes enhanced liposome stability and prolonged *in vivo* circulation [359,360]. Work in the laboratories of Drs. Dewhirst and Needham at Duke University led to the development of low-temperature sensitive liposomes (LTSL) that would later become ThermoDox [361]. This formulation incorporated PEG for steric stabilization and increased circulation. However, the revolutionary change to the formulation design was the incorporation of 10% (mol) lysolipid in order to sharpen the T_m and provide a rapid on-set of membrane permeability, resulting in burst release upon heating [362]. Since this time, many other groups have advanced the field of thermosensitive drug delivery by altering the lipid composition [363,364], loading new drugs into the liposomes [365–367], or by optimizing the heating protocol [368,369]. All of these approaches take advantage of heat-triggered burst release of drugs from thermosensitive carriers, but they also benefit from HT-induced physiological changes such as increased blood flow and increased vascular permeability that increase drug accumulation in tumor (Fig. 4). Current TSL, including ThermoDox, exhibit very fast drug release when heated to temperatures close to the T_m. Recent work from Motamarry et al. demonstrated *in vitro* that at temperatures above 40 °C complete release of doxorubicin occurred within 2 s [370], although a significant portion of drug remains entrapped within the liposomes and is never released. Fast release is important given that mean blood pool residence times within tumors are generally on the order of seconds (i.e. 2 – 10 s) [371]. The ability to trigger significant release within this short period of time allows for significant drug release as soon as the nanomedicines reach the preheated tumor. Drug is thus released in the tumor vasculature and this leads to substantial amounts of extravasation of free drug into the tumor interstitium [372]. Many groups have demonstrated that TSL are able to significantly increase total drug delivery to tumors [372,373] and result in improved efficacy [374,375]. Specific examples are discussed in Section 8.

The power of HT to alter tumor physiology and increase sensitivity to RT has been described in this review. Researchers have actively explored the ability of nanomedicine-based approaches to deliver radiosensitizers to tumors [376,377]. However, traditional nanomedicines are often limited by an inability to deliver radiosensitizers to the most radioresistant cells within the hypoxic core of the tumor. A recent report from Sadeghi et al. examined the *in vitro* feasibility of loading the radiosensitizer pimonidazole into TSL [378]. The same group has also explored the *in vitro* radiosensitizing effects of ThermoDox on human fibrosarcoma (i.e. HT-1080) cells [379]. Given the ability of TSL to increase tumor accumulation of drugs, these formulations incorporating radiosensitizers represent promising approaches to further increase the radiosensitizing effects of HT.

7.2. RT-triggered release

RT-triggered nanomedicines are a relatively immature field of research with few publications [343], but some recent advances indicate potential future applications. Wu et al. prepared 134 nm cysteine-modified G4.5 dendrimers loaded with doxorubicin [380]. Acidic pH (i.e. pH = 5.0) and RT (i.e. 5 Gy) were used to trigger drug release with release being mediated by RT-induced generation of ROS and an alteration in the dendrimer structure as well as disulfide bond cleavage. Release was not rapid compared to many other triggered release systems (i.e. 18% release at 8 h, 48% at 70 h). This is not an intravascular release system, but would be administered at a suitable time prior to RT (e.g. 24 – 72 h). Deng et al. designed doxorubicin-containing liposomes co-loaded with verteporfin and/or gold nanoparticles in order to actively trigger release by RT [381]. Release was triggered by 1, 2, or 4 Gy RT causing verteporfin to generate singlet oxygen, likely causing oxidation of unsaturated lipids and bilayer disruption. This formulation is promising as dramatic improvements in efficacy in mice bearing human HCT 1116 colorectal tumors were observed for RT combined with intratumoral (IT) injection of RT-triggered doxorubicin liposomes compared to either RT or IT administration of traditional doxorubicin liposomes alone. However, the lack of a control group combining RT with either free doxorubicin or traditional doxorubicin liposomes makes it difficult to assess the benefit of triggered drug release at this point. Most recently, Misra et al. developed calcium tungstate ($CaWO_4$) particles coated in PEG_{5k}-*b*-PLA_{5k} that encapsulate paclitaxel [382]. Inclusion of the calcium tungstate core resulted in dose dependent *in vitro* drug release from the nanoparticles at clinically relevant single RT fractions of 2 and 7 Gy compared to particles not exposed to RT. IT administration

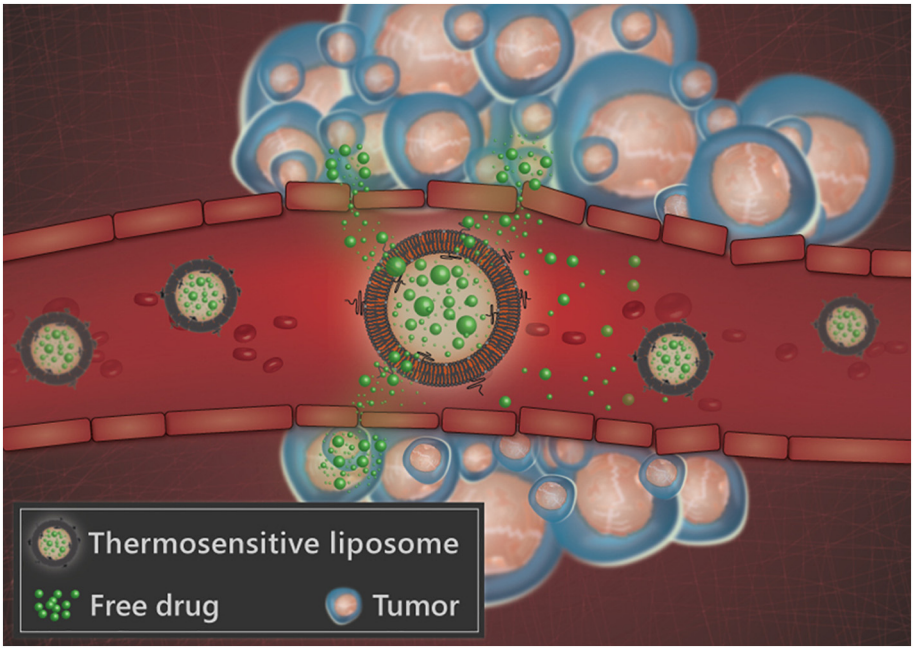

Fig. 4. Heat-triggered intravascular drug release from thermosensitive liposomes. Following the application of HT, drug is quickly and efficiently released from the thermosensitive liposomes within the tumor vasculature. Free drug penetrates from the vasculature into the surrounding tumor interstitium along a concentration gradient.

of the particles with RT resulted in a statistically significant improvement in tumor control and survival compared to RT or the particles alone. This study also lacked a control combining RT with a traditional drug formulation in order to assess the effect of targeting. While the penetration depth and conformal targeting of RT make it an appealing drug triggering strategy, the use of IT administration of these agents suggests a need for development of more stable formulations suitable for intravenous administration.

7.3. pH-triggered release

Many macromolecular drugs have been designed to take advantage of differing pH environments, primarily with the aim of releasing their cargo in response to acidic pH conditions [383]. However, carriers that are designed to release their drug cargo in response to the acidic extracellular conditions of the tumor microenvironment can face difficulties given that more acidic regions of the tumor are also likely to be the least perfused regions. It is more challenging for drug formulations comprised of larger particles to reach these more acidic tumor regions, potentially preventing pH-responsive drug release at the desired location. One of the approaches deemed most promising for development of pH responsive advanced drug delivery systems involves those that provide endosomal escape within tumors [384]. In particular, drug carriers containing genetic material (e.g. siRNA, mRNA) must escape endosomes before being degraded inside lysosomes [385]. Formulations incorporating fusogenic molecules, often lipids, have successfully been developed to accomplish this aim and are now having a clinical impact [386,387]. However, this approach is not related to the acidic tumor microenvironment arising from altered physiological processes and is outside the scope of this review.

HT can trigger release of ChT from TSL, increasing tumor drug accumulation.

8. Potential Impact of HT on Drug Delivery

This review has described the physiological impact HT exerts on tumors. Many of these effects significantly alter the accumulation and distribution of both small molecule and nanomedicine ChT within tumors. Table 1 summarizes these effects and the impact on drug delivery. Specifically, HT is able to increase blood flow, perfusion, and vascular permeability while decreasing IFP, resulting in an increased accumulation of small molecule and/or macromolecular drugs. Additionally, HT can increase the extracellular pH within tumors to values that are closer to the conditions measured in normal tissues. This has a positive impact on the efficacy of many weak base ChT. Finally, HT can trigger release of drug from thermosensitive nanomedicines and further impact drug distribution and accumulation. Each physiological change affects individual drugs in a distinct manner that is dependent on the physicochemical properties of the drug. In addition to molecular weight, factors affecting drug accumulation and distribution include logP, pKa, and water solubility as these parameters influence circulation times, protein binding, vascular permeability, and cellular uptake. Specific examples of HT altering drug accumulation and distribution profiles within tumors are discussed below.

Table 1
Physiological consequences of HT and the effect on small molecule and macromolecular drug delivery.

Physiological Effect		Predominant Impact on Accumulation	
↑	Blood flow	↑	Small/macro
↑	Perfused fraction	↑	Small/macro
↑	Transcytosis vascular permeability	↑	Small molecules
↑	Paracellular vascular permeability	↑	Macromolecules
↓	Interstitial fluid pressure	↑	Macromolecules

Moving from preclinical animal models to clinical treatment has proven to be a particularly difficult transition in the cancer research field. Comparative oncology seeks to bridge this gap by studying spontaneous cancers in companion animals to generate complementary information when combined with traditional preclinical studies [388]. Companion animals represent a diverse population with intact immune systems and complex tumor biology, more comparable to that of humans [389]. The HT field has been advanced by many comparative oncology studies that suggest clinical potential. Furthermore, canine tumour volumes are generally more comparable to those in humans relative to those in mouse, rat, and other commonly used preclinical cancer models. Indeed, in a previously discussed study, Thrall et al. applied MW HT to canine tumors of up to 400 cm^3 in volume [215]. This is particularly important in HT studies, as treatment protocols must be adapted for tumor volume. As a result, canine tumor heating protocols more closely resemble clinical practice. Hauck et al. completed important early stage safety and toxicity studies of ThermoDox in spontaneous canine tumors [390]. ThermoDox was administered intravenously at a dose of 0.7 - 1.0 mg/kg in a dose escalation study. MW HT was applied for 30 min during drug administration and for 1 h afterwards with a maximum median temperature of 44 °C in an effort not to reduce tumor blood perfusion. A maximum tolerated dose of 0.93 mg/kg was identified and an average tumor accumulation of 9.12 ± 6.17 ng/mg tissue was recorded (for 1 mg/kg dose). Matteucci et al. measured the effect of HT on nanomedicine accumulation in spontaneous feline sarcomas [391]. ^{99m}Tc -labeled liposomes, (similar in lipid composition to Doxil) were administered to cats and tumor accumulation was measured by planar scintigraphy. Cats received intravenous liposome injections at normothermic temperatures and then again 48 h later in combination with MW HT (60 min with a median temperature of 44.6 °C). HT increased liposome accumulation in all 14 animals for which measurements could be made. As this was a comparative oncology study, there was tremendous variability in tumor volume (1.2 to 236.2 cm^3). However, there was no correlation between tumor volume and the magnitude of increased liposome accumulation, suggesting broad applicability of this HT regimen to increase nanomedicine accumulation.

8.1. Impact of HT on drug accumulation

Many preclinical studies have demonstrated that HT is able to increase tumor accumulation of a number of different ChT [392]. Medical imaging and/or *ex vivo* tumor resection and drug extraction are the most common measurement techniques used to assess drug accumulation. To accurately determine tumor accumulation, drug within the tumor interstitium can be distinguished from drug in blood by subtracting the blood pool contribution or by removing it entirely by perfusing the vasculature with another fluid (e.g. saline). Microdialysis techniques can also be used to measure drug concentrations directly in tumor extracellular fluid [393]. Several recent preclinical studies have confirmed the viability of these approaches with both small molecules and higher molecular weight constructs. Farr et al. administered free doxorubicin intravenously to mice and the Sonalleve MR-guided high-intensity focused US (MR-HIFU) system was used to heat tumors for 15 min with a mean tumor temperature of 41.2 ± 1.3 °C. 10 min after treatment, the vasculature was flushed with saline and a 2-fold increase in doxorubicin concentration was measured in resected tumors [394]. Jenkins et al recently reported that 60 min of water bath HT at 42.5 °C increased tumor accumulation of the FDA-approved photodynamic therapy (PDT) agent, porfimer sodium, by 3-fold at 24 h following administration [395]. Interestingly, HT treatment did not result in an increase in porfimer concentration in the tumor immediately following heating, indicating that HT altered tumor physiology over a longer timescale. Importantly, in addition to increasing PDT agent accumulation, the addition of HT resulted in a statistically significant

improvement in anti-tumor efficacy of the PDT agent and laser stimulation. Matteucci et al. intravenously administered ^{99m}Tc-labeled liposomes and employed gamma scintigraphy imaging to assess the impact of HT on nanomedicine accumulation in domestic felines [391]. Microwave application of local HT was applied to spontaneous tumors for 60 min and in all 14 cats liposome accumulation was greater in tumors post-HT compared to unheated tumors. This enhanced accumulation persisted without diminishing until the end of the study at 18 h post-administration. Similarly, Kleiter et al. combined administration of Doxil and a tracer quantity of Doxil-like radiolabeled liposomes to determine the effect of HT on liposome accumulation in fibrosarcoma (MCA-R) tumors in rats [396]. The entire tumor volume was maintained between 39 – 44 °C for 45 min and tumor accumulation was assessed by *in vivo* gamma camera imaging, *ex vivo* gamma scintillation counting, and *ex vivo* doxorubicin quantification. HT resulted in a 3- to 4-fold increase in tumor accumulation at both 5 and 18 h. Our recent work demonstrated that HT can increase tumor accumulation of both free drug and nanomedicines. Tumor accumulation of the free anti-cancer agents cisplatin [365], doxorubicin, and alvespimycin [373] increased by 2- to 3-fold following external laser-based application of HT for 25 min at 42.5 °C. The impact of the same HT protocol on nanomedicine application was assessed by computed tomography (CT)-based quantification of iodine-containing liposomes in breast cancer models implanted orthotopically in mice [337]. After subtracting vascular contributions, it was determined that HT significantly increased accumulation by ~2-fold in human MDA-MB-231 tumors, but did not increase tumor accumulation in murine 4T1 tumors. As previously described for this study in Section 6.3.3, the employed HT protocol was able to reduce tumor IFP in MDA-MB-231 tumors, but not 4T1 tumors. This is presumably a significant contributing factor in the impact of HT on nanomedicine accumulation in both tumor models. There is also data that suggests that HT-mediated damage to cells within the tumor microenvironment may increase the extravascular volume fraction available to nanomedicines [397,398]. Furthermore, the timing of HT and ChT administration is an important consideration. Kong et al. have elegantly ascertained that HT (42 °C, 1 h) increased liposome accumulation within tumors for 4 h following HT, but accumulation had returned to baseline after 6 h [399]. Importantly, reheating after 8 h (42 °C, 1 h) did not recapitulate the increased accumulation profile, indicating development of vascular thermotolerance. Frazier et al. measured the effect of MR-HIFU heating (43 °C, 10 min) on the tumor accumulation of both Evans blue dye and 51 kDa Gd-chelated *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers in mice bearing S-180 murine tumors derived from a soft tissue sarcoma [400]. HT resulted in a ~2-fold increase in Evans blue dye accumulation in the tumor at 5 h following treatment. MR-imaging was performed every 15 min for 5 h following injection of Gd-HPMA copolymers and HT treatment in order to accurately determine the temporal effect of HT on accumulation. HPMA copolymers are an important drug delivery platform [401,402] and HT was demonstrated to significantly increase their tumor accumulation over a period of at least 5 h. Interestingly, the Ghandehari laboratory that conducted this research concluded that under these HT conditions (43 °C, 10 min), HIFU increased tumor accumulation to a greater extent compared to plasmonic photothermal therapy HT, which they had previously measured [403]. However, differences in animal and tumor model necessitate further evaluation.

8.1.1. Impact of HT and TSLs on drug accumulation

Delivery of drugs via TSLs combined with HT significantly alters drug accumulation profiles with intravascular release of relatively high concentrations of drug generally resulting in increased tumor accumulation. In 1979, Weinstein et al. were the first to demonstrate the increased tumor accumulation resulting from TSLs and HT [22]. TSLs contained methotrexate and were administered to mice bearing murine Lewis lung carcinomas and HT was applied for 1 h at 42 °C. TSLs and HT resulted in a 4-fold increase in tumor accumulation compared to TSLs without HT and a 3-fold increase compared to free drug and HT. Subsequent studies demonstrated the suitability of using TSLs to increase tumor accumulation of other drugs including cisplatin [404] and doxorubicin [405]. Kong and Dewhirst have summarized the tumor accumulation values for many TSL formulations that preceded the development of ThermoDox. More recently, Hijnen et al. reviewed tumor accumulation literature for studies involving the use of MR-HIFU HT protocols [406]. The majority of the studies summarized by Hijnen et al. used ThermoDox or a similar formulation and most of these studies reported that the addition of HT increased tumor accumulation by 2.5- to 5-fold. Chen et al. demonstrated that ThermoDox has anti-vascular effects [407]. A dose of 5 mg/kg was administered in combination with HT (42 °C, 1 h) to mice bearing human xenograft FaDu tumors. Red blood cell velocity and microvascular density was significantly decreased 24 h following treatment compared to baseline and relevant controls. This is not surprising given the large intravascular concentrations of doxorubicin produced by ThermoDox plus HT and the anti-vascular effects of free doxorubicin [408]. However, these alterations in hemodynamics may have implications for repeat dosing and combinations with other therapies and warrant further investigation.

8.2. Impact of HT on drug distribution

The preceding sections of this review detail the mechanisms through which HT is able to improve the efficacy of RT by decreasing the radioresistance of cells within the tumor that are otherwise difficult to treat. In doing so, HT is able to increase the proportion of the tumor volume for which RT is an effective form of treatment. This paradigm is similar to the manner in which HT improves the efficacy of ChT by increasing the proportion of the tumor that drugs are able to access and treat. This is of importance given that the efficacy of ChT is often limited by heterogeneous distribution throughout the tumor. The distribution issue is of particular importance for antibodies and nanomedicines that have been demonstrated to preferentially accumulate in the periphery of the tumor [409,410] (Fig. 5). Drug distribution of small molecule drugs is also of importance. Trédan et al. reviewed functional impediments to small molecule drug delivery including lack of vascularization and stromal cell barriers [293] that prevent complete eradication of tumors and therefore limit the utility of ChT. Previous work from the same group has observed that the majority of drug that accumulates in tumors is located within several cell diameters of blood vessels [411]. Increasing tumor drug accumulation is only beneficial if more cells within the tumor are exposed to drug and eradicated.

HT is able to improve the drug distribution profile within tumors by delivering ChT to crucial regions that would otherwise not be treated. By increasing the perfused fraction of the tumor and vascular permeability, as well as decreasing interstitial fluid pressure, HT is able to increase tumor accumulation and distribution of intravenously administered ChT. In most tumors, the periphery is the most highly vascularized region and is generally accessible to most forms of ChT. Therefore, most increases in blood flow and vascular permeability will deliver even more ChT to the tumor periphery. While the benefits of this are probably limited, increased drug concentrations of diffusion-mediated small molecule ChT will increase penetration distances of drugs into tumor tissue, providing improvements in drug distribution. HT has also been shown to transiently increase perfusion in the centre of the tumor [93]. This is not surprising, given that HT improves RT efficacy in part by increasing pO_2 in hypoxic regions of the tumor. It is likely that increased delivery of O_2 to these regions would coincide with greater delivery of small molecule ChT.

The principles discussed above, combined with an increase in vascular permeability and a decrease in high tumor IFP, enable HT to improve the distribution of macromolecular ChT. Therapeutic efficacy of long-circulating, traditional nanomedicines is often improved by the addition

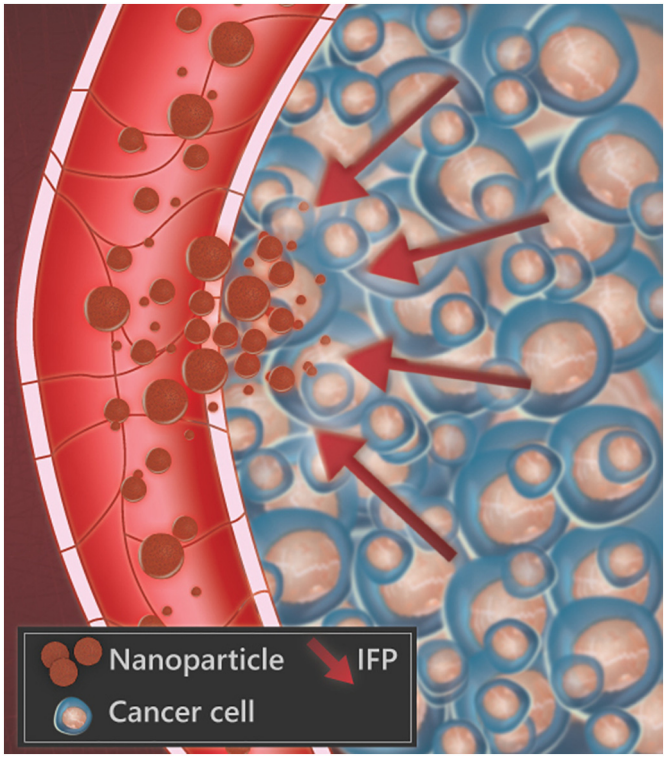

Fig. 5. High IFP prevents macromolecular drugs from accumulating within tumors. Image illustrating the limited distribution of nanomedicines and large MW drugs in tumor tissue with preferential accumulation at the tumor periphery and high IFP precluding homogenous distribution throughout the entire tumor volume.

of HT into the treatment regimen [337,412]. Computational modeling suggests that the biggest impact HT exerts on nanomedicine accumulation within tumors is the result of decreased tumor IFP [337,413,414]. High IFP is a major factor preventing convection-mediated transport of nanomedicines into the tumor interstitium. IFP is commonly higher in the centre of the tumor compared to the periphery [296]. Therefore, by decreasing tumor IFP, HT should be able to preferentially increase nanomedicine accumulation in the centre of the tumor. Our group previously employed CT imaging to demonstrate that HT was capable of lowering tumor IFP and increasing the fraction of the tumor accessible to a Doxil-like imaging agent [337]. Fractions of the tumor were considered to contain significant concentrations of contrast agents when the signal intensity at that time was greater than the mean signal plus two standard deviations for the tumor volume data prior to the administration of contrast [415]. This approach is limited by both the sensitivity of contrast agent detection (i.e. limit of quantification) and the resolution of the medical imaging scanner, but allows for spatial and temporal assessment of contrast agent accumulation in single animals. Despite HT increasing accumulation 2-fold in MDA-MB-231 tumors at the time of maximum tumor accumulation (i.e. 72 h), HT only increased the enhanced tumor fraction at this point by 1.3-fold. However, the kinetics of accumulation were interesting as at early (i.e. 24 h) and late (i.e. 168 h) time points the enhanced volume fraction was higher at 1.7- and 2.2-fold respectively.

8.2.1. Impact of HT and TSLs on drug distribution

The majority of studies examining the effect of HT on the intratumoral distribution of ChT have employed TSL. Several studies have qualitatively demonstrated that HT-triggered release of drug from TSLs is better able to deliver drug to the centre of the tumor compared to conventional ChT approaches. In human squamous cell carcinoma xenograft tumors (i.e. FaDu), Kong et al. qualitatively assessed the distribution of doxorubicin delivered as free molecules, Doxil-like nanomedicines, or TSLs and administered with or without HT (42 °C, 1 h) [374]. Quantitatively, HT increase the tumor accumulation of both traditional and TSLs. Qualitatively, TSLs and HT improved drug distribution such that more drug was observed in the centre of the tumor compared to both free drug and traditional liposomes administered with or without HT. Ranjan et al. employed MR-HIFU HT at 40.5 °C for 30 min in combination with thermosensitive doxorubicin liposomes [416]. In rabbits bearing intramuscular VX2 tumors, they measured a 7.6-fold increase in doxorubicin accumulation for TSLs and HT compared to free drug. They did not quantitatively assess intratumoral distribution profiles, but did note that TSLs combined with HT increased drug accumulation in the centre of the tumor, whereas free doxorubicin administered without HT accumulated solely in the rim. Similar work in a rabbit VX2 tumor model was completed by Staruch et al. [417]. They noted an impressive 27-fold increase in doxorubicin tumor accumulation for heated compared to non-heated tumors 2 h after ThermoDox administration. Greater drug concentrations closer to vessels were noted, but not quantified. In 2013, de Smet et al. measured doxorubicin distribution in *ex vivo* tumor sections by fluorescence microscopy [418]. Doxorubicin delivered via TSLs and HT (42°C, 30 min) was able to enter cells ~50 μm away from tumor vasculature. This was farther than observed for TSLs without HT, and this effect was observed up to 48 h following HT.

Similar to small molecule ChT, TSL-mediated drug delivery is enhanced by HT through increased perfusion. Additionally, intravascular release creates high drug concentrations in blood vessels, potentially increasing drug diffusion distances into the tumor interstitium. Manzoor et al. completed what is probably the most thorough analysis to date of the effect of HT on drug distribution in the tumor [372]. They employed both window chamber models (B16-BL6 murine melanoma tumors) and fluorescence microscopy analysis of histological sections (human FaDu hypopharyngeal carcinoma implanted subcutaneously) and compared the distribution of doxorubicin administered free or encapsulated within traditional or TSLs. Tumors were preheated and then HT was applied for 20 min following intravenous drug administration at average temperatures of ~41.5 °C for both models. Fig. 6 is a striking visual portrayal of the accumulation kinetics of free doxorubicin and TSLs both with and without HT in the melanoma window chamber model with blood vessels visualized in green. For free drug, rapid accumulation and clearance within 5 min can be observed, with greater accumulation under HT. Very little drug leaves the vasculature for the TSL treatment without HT, whereas drug concentration in the tumor interstitium increases over the course of 20 min when HT is applied to mice receiving TSLs. These results were quantitatively validated in 4 – 6 mice per treatment group. Histologic assessment of doxorubicin penetration into the tumor interstitium was performed in the subcutaneous hypopharyngeal model. The addition of HT was found to significantly increase the median penetration distance of free doxorubicin from 29 to 55 μm. Doxil penetration was only measured with HT and was determined to be 34 μm, whereas TSLs combined with HT increased drug penetration distance to 78 μm. It is worth noting that intravascular release generates sustained diffusion of free drug into the tumor interstitium over the course of the HT treatment (Fig. 6), assuming the pharmacokinetic half-life of the nanomedicine is sufficiently long. This constant concentration gradient increases penetration of drug into the tumor interstitium, resulting in increased penetration distances.

Increases in penetration distances from vessels are significant because the drug extravasates radially away from the blood vessel accessing a large volume. For example, the volume fraction accessible to drug extravasating from a 10 μm diameter capillary [419] increases more than 3-fold when HT increases the penetration distance of free doxorubicin from 29 to 55 μm (Fig. 7). The use of TSLs with HT represents a 2-fold increase in the accessible volume fraction compared to free drug with HT.

The tumor accumulation and clearance kinetics of ChT delivered as free drug or TSLs are relatively similar and differ greatly from traditional

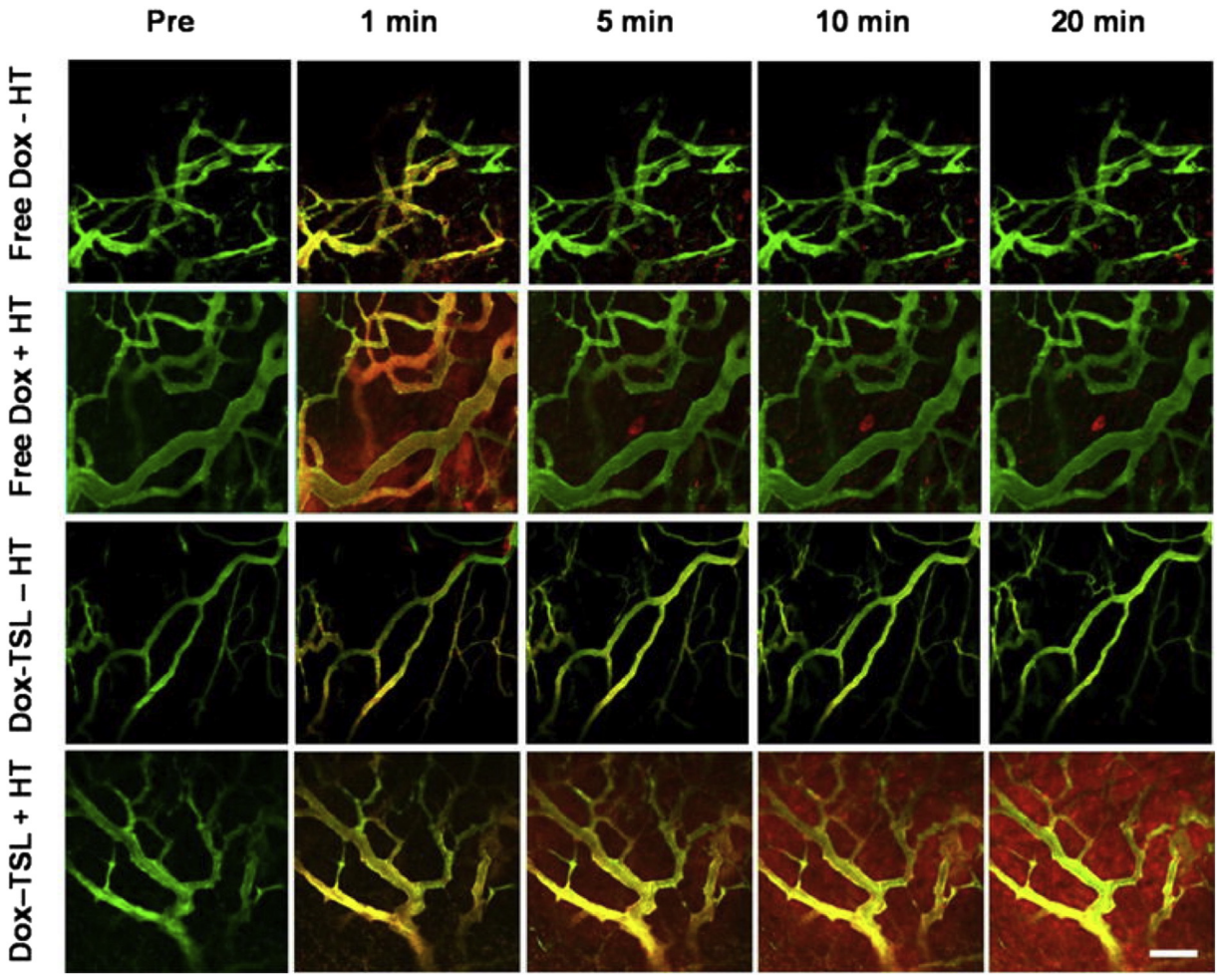

Fig. 6. Confocal microscopy images demonstrating doxorubicin accumulation in tumors. Free doxorubicin or doxorubicin-containing TSLs were administered with and without HT (40.7 – 41.8 °C with 10 min heating prior to and 20 min following drug administration). Mice were implanted with dorsal skin-fold window chamber models of FaDu human squamous cell carcinoma. Blood vessels containing fluorescein-labeled dextran (green), doxorubicin (red), and co-localization (yellow) indicate drug accumulation and clearance over 20 min. Scale bar = 100 μm. Reprinted with permission from [372].

nanomedicine drugs. Most free drugs are rapidly cleared from circulation and maximum tumor accumulation occurs within 0 – 2 h following intravenous administration [372,420,421]. Drugs delivered via thermosensitive carriers are released and accumulate within the tumor primarily within the period of HT treatment that generally lasts for 30 – 60 min [29]. Conversely, traditional long circulating nanomedicines gradually accumulate within the tumor and are retained longer. Maximum drug concentrations usually result around 24 – 72 h following intravenous administration, depending on the half-life and stability of the carrier and retention within the tumor [125]. It is therefore possible to envision the potential benefits of combining both thermosensitive and non-thermosensitive nanomedicines in one treatment regimen that incorporates HT. However, the authors are not aware of any studies that have combined thermosensitive and traditional nanomedicines. It is hypothesized that the different temporal and spatial drug distribution profiles resulting from delivery via traditional and thermosensitive nanomedicines would provide complementary therapeutic benefit to patients. Furthermore, HT should further increase the fraction of the tumor to which both types of formulation are able to deliver ChT. TSLs have a shorter half-life compared to non-TSLs. However, a treatment plan involving co-administration is feasible. TSL-mediated drug delivery is optimally achieved by heating the tumor as quickly as possible following liposome administration (or pre-

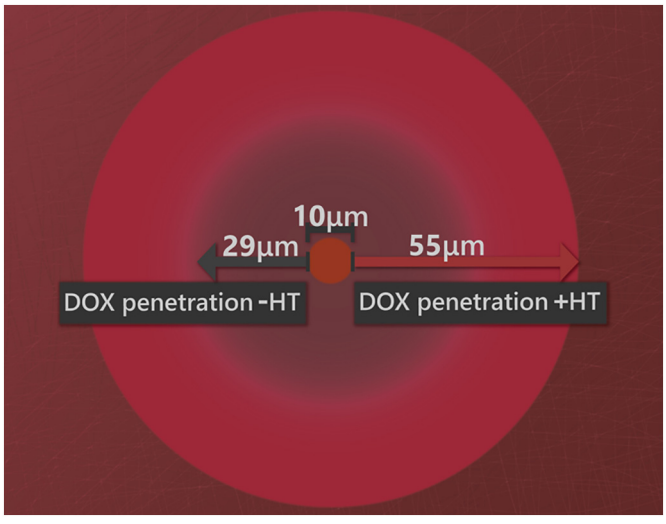

Fig. 7. Increased tumor region accessible to ChT as a result of HT. Schematic representation of the tumor region accessed by drug when penetration distance from blood vessel (diameter = 10 μm) increases from 29 μm (dark purple) to 55 μm (red) with the application of HT (as reported for doxorubicin in Manzoor et al. [372]).

heating of the tumor). The reduction in tumor IFP and vascular changes can benefit traditional nanomedicines after HT has ceased. Given the known synergy between HT and RT as well as some ChT drugs and RT, it is easy to see the benefit of incorporating RT into complex treatment plans in order to provide further benefit to the patient.

HT can increase drug accumulation and distribution, improving ChT efficacy.

9. Conclusion

There is still much research to be conducted in order to provide patients with optimized treatment plans that incorporate HT. While combining HT with ChT or RT has been shown to improve patient outcomes in some studies [12,13], other trials have been less conclusive [422]. The tumor microenvironment exerts numerous negative influences on the therapeutic efficacy of ChT and RT. Several factors that can be affected by HT are discussed in this review. In low pH tumor microenvironments, the cytotoxicity of ChT is often reduced. Low tumor pO_2 significantly reduces the efficacy of RT and many forms of ChT. Elevated tumor IFP is a major impediment to the effective delivery of nanomedicine ChT and also reduces the efficacy of RT. HT treatment of tumors at temperatures ~39 – 42 °C can increase tumor blood flow and perfusion and can also be used to trigger drug release from thermosensitive carriers. Increases in HT-mediated blood flow can increase vascular permeability and reduce IFP in tumors in which it is elevated. The combined effect of increases in tumor blood flow, perfusion, and vascular permeability, along with decreased IFP can significantly increase drug accumulation and distribution within the tumor. The potential combination with HT-triggered drug delivery systems can further enhance drug accumulation and distribution. These improvements are critical as ChT efficacy is primarily governed by the temporal and spatial intratumoral drug distribution with successful treatment dependent on treatment of all cancer cells within the tumor. Furthermore, increased blood flow can remove acidic metabolites and balance extracellular pH to more normal physiological levels, potentially improving the efficacy of ChT that does reach the tumor. RT efficacy can be significantly enhanced by the ability of HT to increase tumor pO_2 and decrease tumor IFP. Through increased delivery of radiosensitizing molecules, HT can provide further benefit to RT.

HT can improve the overall efficacy of both ChT and RT in patients with tumors that have low vascular permeability, low pO_2, low extracellular pH, and/or high IFP. However, due to the difficulty involved in assessing these tumor microenvironment parameters, they are not used as inclusion criteria when enrolling patients in clinical trials involving HT. These trials are therefore not designed to optimize potential clinical success. Inter-patient variability necessitates the inclusion of personalized physiological information about the patient's tumor (e.g. perfusion characteristics, vascular permeability, IFP, pO_2, and both pH_i and pH_e) and their response to HT. Medical imaging is best able to provide a spatial map of these characteristics within the tumor volume, allowing for patient stratification and assessing response to therapy. The future of advanced drug delivery for cancer therapy is personalized medicine that considers the genetic characteristics of the tumor when selecting the most appropriate therapeutic agent. However, personalized medicine must also entail understanding the biophysical characteristics of the tumor. Imaging of these characteristics allows for the rational integration of HT into treatment protocols in order to modulate these biophysical characteristics to enhance ChT and RT and provide individual patients with optimal treatment regimens.

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