Published in final edited form as:

Nanomedicine (Lond). 2011 April ; 6(3): 545–563. doi:10.2217/nnm.10.153.

Nanoparticle Pre-Conditioning for Enhanced Thermal Therapies in Cancer

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Abstract

Nanoparticles show tremendous promise in the safe and effective delivery of molecular adjuvants to enhance local cancer therapy. One important form of local cancer treatment that suffers from local recurrence and distant metastases is thermal therapy. Here we review a new concept involving the use of nanoparticle delivered adjuvants to “pre-condition” or alter the vascular and immunological biology of the tumor to enhance its susceptibility to thermal therapy. To this end, a number of opportunities to combine nanoparticles with vascular and immunologically active agents are reviewed. One specific example of pre-conditioning involves a gold nanoparticle tagged with a vascular targeting agent (i.e. TNF- α). This nanoparticle embodiment demonstrates pre-conditioning through a dramatic reduction in tumor blood flow and induction of vascular damage which recruits a strong and sustained inflammatory infiltrate in the tumor. The ability of this nanoparticle pre-conditioning to enhance subsequent heat or cold thermal therapy in a variety of tumor models is reviewed. Finally, the potential for future clinical imaging to judge the extent of pre-conditioning and thus the optimal timing and extent of combinatorial thermal therapy is discussed.

Keywords

Nanoparticle pre-conditioning; colloidal gold; TNF- α ; thermal therapy; hyperthermia; cryosurgery; cryotherapy; cancer

Introduction

Nanotechnology holds tremendous promise for the treatment of cancer. Recent work has focused on the unique potential of nanotechnology to improve drug delivery, imaging

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Financial Disclosure

The authors declare no competing financial interests.

contrast, and heating of tumors over traditional methods in cancer treatment [1, 2]. In some cases, nanoparticles have multifunctional properties or potential for therapy and diagnostics that has led to a new field entitled “theranostics” [3]. There are also increasing opportunities for combinatorial treatment of cancer with nanoparticles [4]. Several classes of nanoparticles, including liposomes, magnetic and metallic, are now in clinical trials for cancer thermal therapy (Table 1). This report explores the opportunities for nanoparticle based thermal therapy enhancement. Many approaches using nanotechnology are based on the delivery and acute activation of particles to tumors to induce cell death via drug release or thermal cytotoxicity. The work we will review here is distinct in that we refer specifically to a relatively unexplored and exciting opportunity to use nanoparticles to alter the vascular and immunological biology of the tumor (pre-conditioning) to augment the antitumor effects of subsequently applied thermal therapy.

About 1.5 million cases of cancer were projected to be diagnosed in the US in 2009 [5]. The NIH estimates overall costs of cancer in 2008 at \$ 228.1 billion with \$93.2 billion for direct medical costs (all health expenditures) [5]. Of these, the most prevalent are prostate, breast, colorectal (with metastasis to liver) and lung cancer with 146,000–192,000 new cases each expected in 2009. The primary treatments for these diseases have traditionally been surgical resection, chemotherapy and radiation. However, other approaches are needed for patients who are poor surgical candidates and for those whose primary treatment has failed. This has spurred the growth of alternative approaches including probe based thermal therapies, or thermal “ablative” technologies for cancer over the last 20+ years [6].

Thermal therapies include: cryosurgery (freezing), radiofrequency ablation (RFA), microwave, laser, magnetic fluid hyperthermia and high intensity focused ultrasound (HIFU) [6] (Table 2). The advantages of thermal therapies include: focal, repeatable, and minimally or non-invasive application with the ability to use image guidance as recently reviewed in a clinically based text on the subject [6]. Unfortunately, while thermal therapies can destroy tissue, a major clinical limitation to their increased use is local recurrence of disease [7, 8]. Researchers in the field have argued that this is generally due to an inadequate understanding and control of the thermal dose, i.e. the necessary temperature and time of exposure needed to ensure killing of all tumor cells [9, 10]. One way to address this is by selectively and controllably reducing the thermal dose required for cytotoxicity between the diseased tissue and normal tissue using combinatorial approaches involving nanoparticles.

Nanoparticles in Thermal Therapies

In thermal therapies for cancer, nanoparticles have been investigated mainly for novel methods of in situ delivery of thermal energy to tumors, as recently reviewed [11]. These approaches utilize the unique properties of nanoparticles inherent to their size and composition such as optical and dielectric properties, magnetic susceptibility, thermal or electrical conductivity. The techniques are, in general, minimally invasive and aim to achieve superior localized heating of the entire tumor mass while sparing surrounding normal tissues. For instance, magnetic fluid hyperthermia aims to heat tissue in an alternating electromagnetic field using nanoparticles of specific size and magnetic susceptibility. This was originally pioneered by Gilchrist in the 1950s with iron oxide [12], but remains an approach of interest as evidenced by clinical trials in Europe for treatment of glioblastoma multiforme and prostate tumors (See Magnetic Fluid Hyperthermia, Table 2) [13, 14]. More recently, photothermal therapy is a growing field where laser light is used to heat nanoparticles (i.e. spherical, rod, and shell particles) with the intention to selectively destroy cells loaded with nanoparticles. Several novel nanoparticle formulations such as gold nanorods, gold nanocages, carbon nanotubes, etc. are being tested in vitro and in vivo to target tumor cells with the goal of achieving photothermal destruction [15–18]. Von

Maltzahn et al. have recently shown that a single injection of polyethylene glycol coated gold nanorods enabled complete photothermal destruction of human xenograft tumors in mice [19]. In a separate study, Zhou et al. have shown the efficacy of copper sulfide nanoparticles in simultaneous in vivo imaging and photothermal ablation therapy [20]. The real potential of photothermal therapy using nanoparticles is evidenced by the fact that plasmonically active nanoshells are currently in clinical trials in the USA using this technology (See Laser, Table 2) [21]. In addition, recent work with radiofrequency Joule heating of gold nanoparticles at 13.56 MHz seems promising for heating cancer cells at nanoparticle concentrations far below that necessary for effective magnetic fluid hyperthermia; although exceptionally high powered fields (i.e. 600 W generator) are needed [22]. It is worth noting here that, similar to other nanoparticle-based therapies, the success of these approaches is highly-dependent on tumor-specific localization of the nanoparticle using a targeted approach [23].

A separate multi-modal approach using nanoparticles in thermal therapies involves delivery of chemotherapeutics to enhance direct cell injury mechanisms. Traditional combinatorial approaches involving chemotherapy or radiation therapy have been utilized in the past in conjunction with hyperthermia [24, 25]. Mild hyperthermia impacts tumor physiology by increasing tumor perfusion and vascular permeability [26]. These effects along with perforation of tumor blood vessel walls, microconvection in the tumor interstitium, and perforation of the cancer cell membrane induced by mild hyperthermia are all conducive to improved drug delivery to tumor stromal and parenchymal compartments [27]. Preclinical work on this approach has shown considerable promise in synergistic destruction of tumor cells, however, the data from preliminary clinical trials while mostly positive, have varied in demonstrating synergy [28–32]. With advances in nanotechnology research, a natural extension of this approach is to package chemotherapeutics in nanoparticle delivery systems for use in combination with thermal therapies. To date the most successful of these strategies is based on liposomal technologies. New clinical trials that involve liposomes with thermal approaches are emerging rapidly. Perhaps the most exciting extension of this concept is the use of a thermally-sensitive liposome that encapsulates anti-cancer agents and is released locally and selectively where the heat is applied. Ponce et al. report on the conception, development and preclinical studies demonstrating increased efficacy using these nanoparticles (liposomes) loaded with doxorubicin in the setting of hyperthermia for cancer therapy [33]. Goldberg et al. report the first preclinical and clinical use of these nanoparticles in conjunction with RFA [34, 35]. In their pilot clinical study on patients with focal hepatic tumors, they show that a single intravenous dose of liposomal doxorubicin (Doxil) administered 24 hours before RFA of the tumors resulted in increased tissue damage at the site of thermal injury compared to RFA alone [34]. These research efforts have resulted in the commercialization of the nanoparticle, ThermoDox™, and initiation of several clinical trials of combinatorial therapy of ThermoDox™ with hyperthermia or RFA [201]. Emerging work in this area includes thermally-sensitive nanoparticles that release genes (i.e. gene therapy) during hyperthermia [36], or freeze sensitive nanoparticles that release drug locally during cryosurgery [37]. These advancements show the potential clinical utility of nanoparticles to enhance direct destruction of tumor cells with thermal therapy. A new but related concept using nanoparticles functionalized with bioactive ligands to “pre-condition” vascular and immunological mechanisms of tumor destruction *prior to* thermal therapies (Figure 1) has also shown significant potential to enhance outcomes as reviewed below.

Nanoparticle Pre-conditioning of Tumors for Thermal Therapy

We define “pre-conditioning” as the use of a bioactive agent (e.g. TNF- α , arsenic trioxide, interleukins, etc.) to modify the vascular and/or immunological components of the tumor

microenvironment so as to make the tumor tissue more susceptible to a secondary treatment such as thermal therapy. Pre-conditioning of the tumor with bio-conjugated nanoparticles requires an understanding of the in vivo mechanisms of thermal injury and the wound healing response that follows. The tumor microenvironment is a complex milieu resultant of the competing interactions between several pro- and anti-tumorigenic components (cytokines, endothelial cells, inflammatory cells, fibroblasts, extracellular matrix elements) [38]. The goal of pre-conditioning is to shift the balance of these interactions in the anti-tumorigenic direction so as to enhance the outcome of thermal therapies (i.e. increased and controlled tumor cell kill, decreased local recurrence). Several components of the tumor microenvironment are amenable to pre-conditioning by drugs already in preclinical or clinical trials (Table 3).

Selection of Pre-conditioning Agent and Nanoparticle Design

Although a large number and wide variety of potential pre-conditioning agents are available (Table 3), the biomolecule of interest in our recent studies, TNF- α , is an ideal pre-conditioning agent due to its well-established mechanism of action against the tumor vasculature, potential for sensitizing tumor cells to thermal injury, potential for eliciting an antitumor immune response, and documented clinical success in solid tumor therapy in native and nanoparticle form (although currently limited in scope). In vitro studies conducted by our group have shown that pre-treatment with native TNF- α sensitizes various tumor cell lines to thermal (heat and cold) injury [39–41]; however, the effect is more pronounced in microvascular endothelial cells. This suggests that even though direct cytotoxicity of tumor cells may be enhanced following thermal therapy the primary mechanism of pre-conditioning plays out within the tumor vasculature. CYT-6091, a polyethylene glycol (PEG) coated gold nanoparticle tagged with tumor necrosis factor alpha (TNF- α), has been explored by our group to deliver TNF- α to the tumor vasculature and pre-condition the tumor microenvironment for enhancement of thermal (heat and cold) therapies. CYT-6091 has already completed a Phase I clinical trial as a standalone anticancer therapeutic [42, 43]. The significant findings of the trial include lack of dose limiting toxicities or drug related serious adverse events, even at a 3-fold higher dose of 600 $\mu\text{g TNF-}\alpha/\text{m}^2$ than the maximum tolerated dose of native TNF- α [43]. In addition, an absence of a systemic hypotensive response was observed, a major limiting factor that has restricted the use of native TNF- α in the clinical setting to isolated limb perfusion treatment of high-grade sarcomas and melanomas [44]. The base rationale behind attaching the cytokine to a nanoparticle (CYT-6091) is to reduce systemic toxicity and improve the localization of TNF- α to the tumor tissue. In several preclinical tumor models, we have shown that a *single systemic injection* of CYT-6091 4 hours prior to thermal therapy results in enhanced therapeutic outcome similar to that of previous preclinical work [45], yet with little to no overt toxicity. The toxicity profile of CYT-6091 may be further improved by the addition of a tumor targeting moiety to the gold nanoparticle such as antibodies, peptides or aptamers. This approach, especially using antibodies to target tumor cell receptors, has been widely tested in various cancers and would reduce the exposure of healthy tissues to TNF- α thereby improving its toxicity profile [23]. This nanoparticle design should allow entry in the tumor via the enhanced permeability and retention (EPR) effect and binding onto the tumor cells. A potential caveat of using nanoparticles to pre-condition tumors for enhanced thermal therapies is the ability of the nanoparticles to directly modify the thermal properties of tumor tissue. This is an area of intense research in the nanotechnology community but only a limited number of studies have been conducted to investigate this behaviour of nanoparticles in biological tissues. In one study, Liu and colleagues conducted experiments that suggested an increase in thermal conductivity of ex vivo tissues when injected locally with a large amount of iron oxide nanoparticles (estimated at 5% local particle volume fraction) [46]. In the case of gold nanoparticles, a recent study by Shalkevich et al showed

no significant increase in the thermal conductivity of gold nanoparticles suspended in water even at particle volume fraction of 0.11% [47]. These results indicate that incredibly high volume fractions of nanoparticles are required within tumors to affect its thermal properties. Our biodistribution results for CYT-6091 yield tumor particle volume fractions on the order of $10^{-5}\%$ when injected intravenously [48]. Similar particle volume fractions on the order of 10^{-8} – $10^{-5}\%$ were obtained in another study using 20–100 nm diameter neutral PEG coated gold nanoparticles [49]. Thus, we can safely assume that changes in the thermal properties of tumors by nanoparticles do not contribute to the thermal sensitization obtained by the pre-conditioning mechanisms described below.

Pharmacokinetics and Biodistribution

The use of nanoparticles for pre-conditioning the tumor is dependent on delivery of the molecular payload to the tumor site. A limited number of studies have been conducted to determine the pharmacokinetics and biodistribution of gold nanoparticles in vivo. These studies usually look at the effect of size, shape, charge, coating and targeting moiety on the biodistribution of nanoparticles. De Jong et al. studied the effect of size on the biodistribution of gold nanoparticles in naïve rats at 24 hours after intravenous injection [50]. Their results showed that the highest accumulation of nanoparticles occurred in the liver with the 10 nm nanoparticles showing widespread accumulation in various organs. Another study of gold based core-shell particles showed that the maximum tumor accumulation occurs at 24 hours whereas the overall highest accumulation occurs in the spleen (up to 24 days) [51]. More recently, Perrault et al. conducted an elaborate study of the effect of particle size (20–100 nm) and surface chemistry (amount of PEG) on the pharmacokinetics and biodistribution of PEG coated gold nanoparticles [49]. In that study, athymic nude mice implanted with human breast cancer xenograft tumors were used to systematically examine how particle design can be optimized towards efficient tumor targeting. The authors of the study note that two desirable properties of the nanoparticles for tumor targeting - small hydrodynamic diameter and long half-lives - appear to converge when particles below 50 nm are protected with a PEG layer of moderate molecular weight (MW 5 kDa). To support this claim, they present results on the permeation of different sized PEG coated gold nanoparticles through the tumor interstitial space with the smallest diameter (20 nm) particles migrating furthest away from the tumor vasculature (Figure 2B). These and a host of other studies, mainly using liposomal nanoparticles, have led to three major insights into in vivo biodistribution of nano-sized agents: 1) smaller particles (20–200nm) are readily taken up in the tumor due to leaky vasculature (EPR effect); 2) surface modification with hydrophilic polymers (PEG) can reduce uptake by reticuloendothelial (RES) organs (such as liver and spleen); 3) attachment of the targeting moieties on the particle surface results in selective (but not exclusive) uptake by the target organs or tumors [52]. These insights were incorporated in the development of CYT-6091 (a 30 nm diameter colloidal gold nanoparticle coated with PEG and tagged with TNF- α) and resulted in promising biodistribution results obtained in preclinical studies [53].

In our recent studies, a certain degree of tumor targeting of CYT-6091 was verified through pharmacokinetic and biodistribution studies (Figure 2) [48]. A single intravenous injection of CYT-6091 resulted in preferential accumulation of bioactive TNF- α in human prostate xenograft tumors (LNCaP Pro 5) grown in the hindlimb of nude mice within 4 hours as compared to the liver and spleen, the major organs of the reticuloendothelial system that play a vital role in nanoparticle clearance. We also detected that the presence of TNF- α influenced the biodistribution of CYT-6091 by extending the blood circulation time of the gold nanoparticle with reduced uptake in the liver 4 hours after injection [48]. Importantly, loading of the nanoparticle in tumor tissue was also significantly increased by the presence of TNF- α on the surface of the injected particles (Figure 2A, 2C). These findings are in line

with previously published data [53]. We have also conducted in vitro studies using confocal Raman microscopy to non-invasively image the uptake of CYT-6091 by LNCaP tumor cells revealing unique spectroscopic features corresponding to the intracellular localization of GNPs over 2 to 24 hours at the membrane, cytoplasm or nucleus (Figure 2D) [54]. These results established that targeted delivery of TNF- α to tumors by CYT-6091 was possible with limited adverse side effects while also setting the timeframe over which pre-conditioning of the tumor may occur prior to thermal therapy. We have focused much of our recent attention on this particle to develop improved combined treatment strategies since it encompasses feature that we hypothesized would be ideal for sensitization of tumors to subsequent thermal exposures and is progressing in clinical testing (Phase II). There is therefore significant potential to translate the pre-conditioning approach to the clinic in the relatively near future.

Vascular Pre-conditioning by TNF- α or other agents

Systemic delivery of TNF- α through the bloodstream likely leads to its interaction with TNF receptor 1 (TNFR-1) on the luminal side of tumor-associated endothelial cells, setting off a cascade of intracellular molecular interactions that are grouped into two major pathways: NF κ B-activated pro-inflammatory pathway and caspase-mediated apoptotic pathway. In vivo inhibition studies have revealed that the NF κ B-activated pro-inflammatory pathway in endothelial cells is critical for pre-conditioning the tumor with native TNF- α to enhance thermal therapy [39]. These pro-inflammatory changes result from de novo gene transcription and translation of proteins mediated by NF κ B and require around 2 to 6 hours to reach peak response [55]; thus, setting the 4 hour timeframe to obtain effective pre-conditioning. The pro-inflammatory changes induced by TNF- α include vascular hyperpermeability, increased expression of cell adhesion molecules and chemokines (ICAM-1, VCAM-1, E-selectin, P-selectin, CXCL8), recruitment of leukocytes (neutrophils, monocytes), leukocyte-mediated endothelial cell injury, increased synthesis of tissue factor, increased procoagulant activity and decreased anticoagulant activity (Figure 3A–B, 4) [44, 55, 56]. Consistent with these results, Farma et al. measured the rate of interstitial accumulation of an intravenously administered fluorescent marker in TNF- α sensitive and resistant tumors and showed that CYT-6091 induced a selective and rapid alteration of permeability of the tumor vasculature that was tissue factor dependent [57].

Such vascular changes may also be elicited by other potential pre-conditioning agents (Table 3). For example, vascular destructive agents (VDAs) target endothelial cells and pericytes of established tumor vasculature to cause complete tumor vascular shutdown. We have demonstrated that the FDA-approved anti-leukemic agent Arsenic Trioxide has such effects on the microvasculature of solid tumors [58–61]. This likely occurs due to severe oxidative stress and cell death in the vasculature upon exposure to arsenic which leads to vascular stasis and sensitivity to thermal therapy. One subset of VDAs, tubulin-binding agents (e.g. CA4P), disrupt endothelial cytoskeleton or interfere with cell-to-cell contact leading to increased vascular permeability, exposure of basement membrane and induction of the coagulation cascade resulting in thrombosis. Another subset of VDAs, flavonoids (e.g. DMXAA), have a more complex mechanism of action involving DNA damage to endothelial cells inducing apoptosis and upregulation of NF κ B pathways similar to the purported mechanism of action of CYT-6091 leading to tumor vascular shutdown [62]. These VDAs are already in phase I and II clinical trials for cancer therapy and represent ideal first-line candidates for tagging on to nanoparticles for pre-conditioning tumors prior to thermal therapies [62, 63]. Other well-studied molecules/drugs such as vasoactive agents (bradykinin, prostaglandins, leukotrienes, etc.) that induce vascular permeability or pro-coagulant molecules (thromboxane, tPA, etc.) that induce vasoconstriction and thrombosis could also be tested as pre-conditioning agents when tagged on to nanoparticles for selective

and non-toxic delivery to tumor vasculature (See Table 3). Many of these agents do not exhibit their activity on the tumor vasculature alone. Instead, immunological reactions to the cell death occurring in the tumor (such as leukocyte recruitment due to the damage response by the vasculature) often appears to be an intrinsic component of their mechanisms of action. Systemically administered native TNF- α or CYT-6091 may activate circulating blood cells such as neutrophils which would increase their recruitment to the tumor vasculature [64]. These pro-inflammatory changes in the host and the tumor act synergistically with vascular (coagulation and thrombosis) responses after thermal therapy-induced tissue damage to contribute to the enhanced tumor destruction observed after these combined treatments.

Immunological Pre-conditioning

Ongoing research in our group has implicated that neutrophil-endothelial cell interaction following combined nanoparticle and thermal therapy leads to endothelial cell apoptosis that further enhances the ultimate antitumor effects of vascular pre-conditioning [65]. However, it is unclear if the role of neutrophils in tumor destruction ends here. There is evidence to suggest that neutrophils play a larger role in causing direct tumor cell death as well as in modulating the downstream antitumor adaptive immune response [66]. The dual nature of immune cells in relation to the tumor microenvironment is well-recognized and studied in macrophages and T lymphocytes [67, 68]. Thus, there exists an opportunity for immunological pre-conditioning of the tumor which could involve polarization of tumor-associated macrophages to enhance tumor kill or by promotion of cytotoxic T lymphocyte mediated tumor destruction. This type of pre-conditioning could act in concert with vascular pre-conditioning mechanisms as may be the case with CYT-6091. TNF- α is known to promote antitumor immune responses and current research in our group is geared towards understanding the role of TNF- α and CYT-6091 in modulating the adaptive immune response and long term wound involution following thermal therapy [69]. Several other immune modulating biomolecules (Table 3) have shown potential to act as pre-conditioning agents as well. For example, a variety of cytokines (e.g. IL-2, IL-12, IFN- α in addition to TNF- α) are in clinical trials as antitumor therapeutics especially in combination with chemotherapy (Table 3).

Anti-CTLA4 antibodies are another particularly exciting prospect as pre-conditioning agents because they have been extensively researched and are also already in clinical trials though not in combination with thermal therapies. These antibodies are known to successfully inhibit the immunosuppressive environment of tumors to elicit an effective antitumor immune response however their clinical use is limited by autoimmunity side effects due to the high systemic dose required [70]. To overcome this limitation, Lei et al. have tested a nanoparticle system loaded with anti-CTLA4 antibodies in a preclinical tumor model showing increased antitumor activity with decreased toxicity compared to native antibodies [71]. Other immune modulating agents such as TGF- β inhibitors and toll-like receptor (TLR) agonists are also gaining immense clinical interest as antitumor therapeutics (Table 3). By taking into account the mechanisms of thermal injury (heat and cold) in their selection, a wide variety of vascular and immunological pre-conditioning agents with near term potential would be expected to have great potential to be combined with nanotechnology to enhance thermal therapies in cancer and avoid the typical side effects encountered with each type of agent when used systemically on their own.

Mechanisms of Thermal Injury

The vast amount of literature on thermal therapies differentiates between three forms of thermal (heat and cold) injury in tumor tissues: direct cell injury, vascular injury and immunological injury [72–74]. In cold-based therapies (i.e. cryosurgery), the tissue is frozen

usually to a cryogenic (i.e. $< -40^{\circ}\text{C}$) temperature. In this process, damage to cells typically occurs by a phase change event of intracellular or extracellular water (liquid to solid) causing either extreme dehydration or intracellular ice formation [72]. This can in turn lead to biochemical events such as protein denaturation or peroxidation of lipids that terminate in necrosis or apoptosis of the cells [72, 74]. In a distinct but related manner, heating of cells yields phase change within the macromolecules (protein, DNA, lipids) of cells themselves. However, most of the research evidence points to protein denaturation as the rate limiting step in direct cell injury due to heating [75]. If these events are sub-lethal there is usually heat shock response that can interact with the proteins and lipids and keep the cell alive, however, once the injury is too severe cell death occurs again by necrotic or apoptotic mechanisms [75]. In the case of both heat and cold direct cell injury, the end result is membrane destabilization and hyperpermeability [72, 73]. Pre-conditioning agents that decrease the threshold for hyperpermeability of cell membranes could thus improve nanoparticle uptake and facilitate delivery of cytotoxic drugs via nanoparticles into tumor cells. Microenvironmental factors such as tumor blood flow, pH and oxygenation are known to affect the thermosensitivity of tumor cells and thus present an opportunity for modulation by pre-conditioning agents to increase the thermosensitivity of tumor cells [73]. For example, blood flow acts as a heat sink and source in hyperthermic and cryotherapy respectively hindering the attainment of lethal end temperatures within the tumor tissue during thermal therapy. Direct cell injury is an important mechanism of thermal injury; however, in vivo it is supplemented by vascular injury mechanisms that extend the thermal damage. During cold-based therapies, microvascular shutdown usually occurs at -20°C after moderate cooling and thawing rates in a variety of tissues, although this can occur at slightly higher temperatures in tumors [72]. Similarly, hyperthermia even at mild supraphysiological temperatures can cause microvascular dysfunction and shutdown in tumor tissues [26]. One possible difference between heat and cold injury is the amount of tumor tissue that undergoes reperfusion after the initial thermal insult. Blood flow, which will cease during the freezing event, is usually re-established after thaw. However, after thermal therapy only the volume of tumor tissue exposed to lower hyperthermic temperatures ($<50^{\circ}\text{C}$) can undergo reperfusion due to the central part of the thermal lesion exposed to higher temperatures being coagulated or thermally fixed [76]. This mechanistic difference may affect ischemia-reperfusion injury differentially in thermal injury by heat versus cold based therapies. It is now well-established that vascular injury progresses beyond the initial thermal insult (both heat and cold) and typically peaks at day 3 following thermal therapy [72, 73]. Tumor endothelial cell damage is the hallmark of vascular injury leading to increased vascular leakage, vessel wall adhesiveness and coagulation and thrombotic events within the tumor vasculature [73]. This progressive cessation of blood flow leads to ischemia within tumor tissue and necrosis of tumor cells. This mechanism of thermal injury is exploited in the use of CYT-6091 as a pre-conditioning agent and serves as the rationale for exploring other vascular pre-conditioning agents (Table 3) for use in combination with thermal therapies. The progression of vascular injury in the thermal lesion is also accompanied by recruitment of inflammatory cells (neutrophils) that could be responsible for the progressive nature of vascular damage or may be directly exerting cytotoxic effects on remnant viable tumor cells within the thermal lesion [66, 77, 78].

The final, relatively poorly understood, mechanism of immunological stimulation/injury in thermal therapies is based on the premise that thermal injury activates a host-mediated antitumor immune response. Sabel provides a thorough review on the conflicting set of preclinical studies examining the immune response to cryosurgery [79]. The immune response following hyperthermia is also actively being studied [80]. However, there is still a lack of clear understanding of the exact mechanisms of the immune response following thermal therapies partly due to distinct differences observed when time/temperature history and local versus whole body heating are considered. There is evidence to suggest that

necrotic cells releasing 'danger signals' are more efficient at inducing an immune response than apoptotic cells [81]. Thus we may hypothesize that thermal therapies resulting in an increased fraction of necrotic versus apoptotic cells would be more amenable to immunological injury. It is also conceivable that certain thermal therapies may induce a greater release of 'danger signals' and thus a stronger antitumor immune response from the host which could be yet another way to differentiate between heat and cold based therapies. For example, the coagulative and thermally fixed zone formed in high temperature therapies could prevent the release and exposure of danger signals to the host immune cells indicating possibly leading to a variable immune response compared to cold based therapies. Various studies have demonstrated synergistic antitumor effects when combining immunotherapy and thermal therapies (both heat and cold) (Table 3). In general, there is much still to be learned about the role of inhibitory elements of the immune system and the induction of effective antitumor immunity by thermal or other methods. It seems imperative that the immune portion of the antitumor effect of vascular damaging nanoparticle and thermal therapy delivery strategies must be accounted for by using immunocompetent models as much as possible or in addition to xenograft models.

We have demonstrated numerous positive tumor responses in our models with nanoparticle pre-conditioning for improved hyperthermia and cryosurgery in both immunocompetent and immunocompromised models (Table 4).

Nanoparticle Enhanced Hyperthermia

The first demonstration of nanoparticle pre-conditioning enhancement of tumor thermal therapy was conducted by Visaria et al. [41]. In this work, SCK hindlimb tumors grown in A/J mice and FSaII hindlimb tumors grown in C3H mice were injected with a single intravenous dose (250 μ g TNF- α /kg) of CYT-6091 4 hours prior to heating for 60 min at 42.5°C using a water bath. These preclinical studies in mice showed significant increases in heat-induced tumor growth delay with CYT-6091 pre-conditioning prior to hyperthermic treatment (Figure 3C) [40, 41]. Up to 80% reduction in tumor blood flow, as assessed by uptake of the radioactive tracer rubidium, was also measured at 4 hours after CYT-6091 injection that is consistent with known localized mechanisms of TNF- α vascular effects and damage in tumors (Figure 3A–B). This accentuation of hyperthermic injury also shows clear dose dependency. Our group has also conducted preliminary translational studies using VX2 tumors grown in rabbit kidneys. Administering CYT-6091 prior to RFA yielded a significantly larger zone of central necrosis (i.e. 23% increase in ablation volume) than RFA treatment alone on histological evaluation 3 days after the procedure. Control tumors that were not treated with RFA also showed a significant reduction in tumor volume after CYT-6091 treatment alone although not as much as RFA plus CYT-6091 group [82, 83]. This reduction in tumor volume after CYT-6091 treatment alone within 3 days usually occurs after multiple injections over time and may be attributed to the unique nature of the VX2 tumor physiology (i.e. more susceptible) [43]. Typically, our in vivo studies in mice yield significantly less tumor growth delay when CYT-6091 is administered without thermal therapy [40, 41]. Clinically, a multiple dosing schedule was used in a Phase I trial in which CYT-6091 was administered as a standalone cancer therapeutic [42, 43].

Nanoparticle Enhanced Cryosurgery

We have also extensively investigated CYT-6091 particles for pre-conditioning tumors to enhance cryosurgical injury in preclinical models. Cryosurgery was conducted using a Joule-Thomson based argon cryoprobe with the tip temperature maintained at -100°C . Systemic injection of 200 μ g TNF- α /kg of CYT-6091 4 hours prior to cryosurgery of human prostate cancer xenograft (LNCaP Pro 5) and uterine fibroid (ELT-3) tumors grown in hindlimb of nude mice resulted in significant tumor growth delay and in some cases

complete remission compared to mice injected with an equivalent dose of native TNF- α or the control nanoparticle without TNF- α (Figure 3C, 5) [84, 85]. Furthermore, mice administered with CYT-6091 displayed no overt toxicity whereas nearly 25% of mice injected with native TNF- α prior to cryosurgery died within several hours after the therapy. Since tumor kill by cryosurgery is highly dependent on the temperature and rate of temperature change experienced by the tumor, in subsequent studies we used the dorsal skin fold chamber (DSFC) tumor model coupled with real time infrared imaging and thermocouple measurements to obtain the spatial temperature profile of the tumor tissue during and immediately after cryosurgery. Similar to hyperthermic injury, we also discovered a dose-dependence of CYT-6091 for enhancement of cryosurgical injury [84, 85]. In a pilot translational study using VX2 tumors grown in rabbit kidneys, we also obtained a significant decrease in the rate of peritoneal carcinomatosis (metastases) in animals treated with cryosurgery and CYT-6091 compared to cryosurgery alone [82]. This was an unexpected finding and further investigations are underway in preclinical tumor models to interpret whether this was a direct effect on mechanisms of tumor spread or a stimulation of antitumor immunity.

Potential for Enhanced Imaging by Nanoparticle Pre-Conditioning

While we have shown that nanoparticle pre-conditioning is capable of enhancing both hyperthermic and cryosurgical injury in solid tumors, another important potential of this concept lies in its ability to enhance the clinical imaging techniques associated with thermal therapies. For instance, pre-conditioning events can be imaged to provide longitudinal monitoring of the state of pre-conditioning to optimize the time of administration of the secondary treatment. We have already shown that the administration of CYT-6091 is accompanied by a significant 80% reduction in tumor perfusion [40, 41]. Hence, current imaging technologies such as computed tomography (CT), magnetic resonance imaging (MR) or even contrast enhanced ultrasound (CEUS) may prove useful in measuring perfusion drops associated with pre-conditioning that signal an optimal timing for thermal therapy [86, 87]. Similarly, the development of myeloperoxidase-specific imaging agents offers the unique opportunity of monitoring the tumor biology before and after therapy by imaging the recruitment of neutrophils into the tumor tissue [88, 89]. Recent data collected in our lab has shown, on histology, the presence of a neutrophil rich inflammatory infiltrate that peaks at day 3 after TNF- α enhanced cryosurgery (Figure 6A–B) [65]. In this work, LNCaP tumors were grown in DSFCs implanted on nude mice. Tumors were pre-conditioned using topical administration of 200 ng native TNF- α 4 hours prior to cryosurgery. Intravital imaging, thermography, and post-sacrifice histology and immunohistochemistry were used to longitudinally assess iceball location and the ensuing biological effects (e.g. neutrophil infiltration) after cryosurgery with and without TNF- α pre-treatment within the DSFC tumor tissue. In a separate experiment using TRAMP-C2 tumors grown subcutaneously in nude mice, we have also shown that this recruitment of neutrophils to the tumor site post cryosurgery can be non-invasively imaged using luminol bioluminescence imaging (Figure 6C, unpublished results). Based on these preliminary findings, we speculate that the enhancement of neutrophil recruitment by nanoparticle pre-conditioning can be imaged and quantified using myeloperoxidase-specific imaging agents. Additionally, since we have shown that these pre-conditioning events (perfusion drop and neutrophil recruitment) persist and are enhanced after thermal therapy, we hypothesize that they may be used to quantify treatment effects and serve as prognostic indicators of the outcome of thermal therapy. This hypothesis is the basis of ongoing work in our group.

Nanoparticle pre-conditioning can also be used to predict the lethality of the thermal therapy in the case of cryosurgery. Many clinical imaging technologies including ultrasound, CT and MR easily distinguish the edge of the cryosurgical iceball in vivo [90]. Unfortunately, the

current guideline for cancer destruction by freezing requires temperatures below a critical -40°C isotherm [91, 92]. This is achieved in less than 50% of the total 3D iceball volume, assuming the iceball to be roughly elliptical and the probe temperature to be roughly -160°C [93]. It has understandably led to confusion about how much of the tissue and/or cancer to freeze during cryosurgery. This confusion persists today and undoubtedly contributes to under and over freezing that yield local recurrence and complications respectively. With nanoparticle pre-conditioning, we have shown in the DSFC model that we can move the tissue-destruction isotherm to -0.5°C , suggesting that cryosurgical destruction can be effectively doubled and intra-operative imaging of the iceball correlates to destruction, since in such conditions, “what you see (iceball) is what you get (destruction)” [84, 94]. This finding also translated to the hindlimb model in which cryosurgery was performed until the edge of the iceball overlapped with the visible edge of the tumor yielding apparent remission in a significant percent of animals (Figure 5) [84]. These data suggest the clinical use of a nanoparticle system, such as CYT-6091, could enable the surgeon for the first time to visualize the zone of tumor destruction by simply imaging the ice formation during cryosurgery.

Conclusions

The concept of pre-conditioning the tumor microenvironment using nanoparticles is relatively new and many avenues of basic and clinical research particularly for use in the field of thermal therapy enhancement remain largely open. Our group has demonstrated the use of a gold nanoparticle system (CYT-6091), which has recently completed a Phase I clinical trial and moved into Phase II as a standalone cancer therapeutic, to pre-condition a variety of preclinical tumor models prior to thermal therapies (using both heat and cold) (Table 4). Pharmacokinetic and biodistribution studies of CYT-6091 have shown tumor-specific drug and nanoparticle localization and we have demonstrated that a single systemic injection of the nanoparticle at a critical time period (4 hours) prior to thermal therapy suffices to pre-condition the tumor with no overt toxicity. Further, vascular (perfusion defect) and immunological (leukocyte recruitment) mechanisms responsible for pre-conditioning using CYT-6091 have been demonstrated by various techniques and suggest imaging as a possible clinical tool for assessment of the optimal time for subsequent thermal therapy intervention. Thermal therapy outcomes after appropriate pre-conditioning with CYT-6091 have been able to induce tumor regression without toxicity (Table 4, Figures 3, 5), establishing the validity of this clinically realistic approach.

Future Perspective

The constantly evolving and heterogeneous nature of cancer has resulted in a shift from single agent therapy to multi-drug regimens and combinatorial therapies for improved local and metastatic control. This is particularly true for thermal therapy in cancer which is currently dominated by regional hyperthermia combined with standard-of-care chemotherapy and/or radiotherapy for a wide variety of tumors. More recently, focused minimally invasive thermal therapy approaches such as cryosurgery, microwave, and radiofrequency ablation (Table 2) are also finding favor with clinicians and several clinical trials are in progress to test the efficacy of combinatorial therapy using these techniques with chemotherapeutics. A small subset of these trials involves thermal therapies with commercially manufactured nanoparticles (Table 1). This subset of clinical trials represents the successful realization of preclinical research efforts over the past decade in nanomedicine and heralds a new future in cancer therapy. Some of the key challenges going forward are: (1) selection of the appropriate nanoparticle, pre-conditioning agent and thermal therapy for the specific tumor type; (2) demonstration of selective nanoparticle delivery, safety and efficacy and expanded understanding of nanomedicine toxicological

considerations; (3) timing and guidance of thermal therapy perhaps by imaging pre-conditioning after nanoparticle administration; and (4) controlling the extent of thermal intervention necessary to kill the tumor. As we await future nanoparticle development and optimization, even today there may be few barriers to the use of existing manufactured nanoparticles (i.e. CYT-6091) to pre-condition and treat solid cancers with thermal therapies in Phase I/II clinical trials.

Acknowledgments

This work was supported by funds from the National Institutes of Health (project#: R01CA075284 and R01CA44114) and Institute for Engineering in Medicine (University of Minnesota). The authors thank Raghav Goel and Rachana Visaria for valuable discussions and preliminary work on the pre-conditioning concept. CYT-6091 nanoparticles were a kind gift from CytImmune Sciences, Inc (Rockville, Maryland, USA). Special thanks to Warren Chan and Mehmet Toner for useful discussions about this work.

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Figure 1. Nanoparticle Pre-conditioning of Tumor

Illustration of the concept of pre-conditioning the tumor using a bio-conjugated nanoparticle prior to thermal therapy for achieving enhanced tumor destruction. Systemic injection of the nanoparticle leads to its accumulation in the tumor within a short timeframe (minutes-hours). Localization of the nanoparticle within the tumor initiates vascular and immunological pre-conditioning events (detailed in text) that reach peak response in a longer timeframe (hours-days). Thermal therapy (heat or cold) is then administered at an optimal time based on nanoparticle pre-conditioning to achieve superior tumor destruction over thermal therapy alone.

Figure 2. Nanoparticle Uptake in Cancer during Pre-conditioning

(a) Control NP and CYT-6091 accumulation in prostate tumors based on atomic emission spectroscopy (AES) measurements of gold. Presence of TNF- α on the nanoparticle increases its uptake in the tumor [48]. Results are expressed as mean $\pm$ SD. (b) 8 hours post injection, 20 nm control nanoparticles migrate far into the interstitial space of the tumor from blood vessel (arrow) [49]. (c) Electron micrograph of prostate tumor 3 days post injection shows CYT-6091 NPs scattered around in tumor individually or in membrane bound organelles (arrows). (d) In vitro uptake analysis of LNCaP (prostate cancer) cells with confocal Raman microscopy shows no internalization of CYT-6091 at 2 hours (top) incubation whereas perinuclear localization (bottom, particles in green) after 24 hours of incubation. Quantitative analysis of cellular uptake via AES shows increasing cellular uptake over time (right).

Figure 3. Thermal Therapy Enhancement by Pre-conditioning with TNF- α Tagged Gold Nanoparticles

(a) Blood perfusion changes within SCK mammary carcinoma tumors assessed by uptake of radioactive rubidium in tumors after CYT-6091, TNF- α and Control NP systemic injection [40]. CYT-6091 produces the maximum decrease in tumor blood perfusion 4–6 hours after systemic injection. Results are expressed as mean $\pm$ SEM and normalized to untreated controls. * Indicates statistically significant difference from untreated control. (b) H&E stain of a TRAMP-C2 tumor grown in a dorsal skin fold chamber, 4 hours after topical administration of native TNF- α , showing thrombus formation and neutrophil margination in blood vessels. (c) Tumor growth delay data with and without CYT-6091 pre-conditioning in

pre-clinical tumor models: FSaII fibrosarcoma & SCK mammary carcinoma with heat [40, 41] and LNCaP prostate carcinoma & ELT-3 uterine leiomyoma with cryosurgery [84, 85]. Results are expressed as mean $\pm$ SEM and normalized to control tumor volumes at Day 10 after therapy. In some cases, Day 10 volumes were linearly extrapolated from adjacent data points. * Indicates that Day 10 data is not available as tumors completely regressed by Day 4 [40].

Figure 4. Proposed Mechanism of Pre-conditioning with TNF- α Tagged Gold Nanoparticles
 Schematic showing interaction of CYT-6091 with tumor endothelium and cells leading to pro-inflammatory pre-conditioning events i.e. vascular hyperpermeability, recruitment of leukocytes (neutrophils, monocytes, lymphocytes), leukocyte-mediated endothelial cell injury, increased synthesis of tissue factor, increased procoagulant activity and decreased anticoagulant activity.

Figure 5. Cryosurgical Destruction of LNCaP Hindlimb Tumors

200 µg TNF- α /kg of CYT-6091 was intravenously administered 4 hours prior to cryosurgery of LNCaP hindlimb tumors grown in nude mice. Cryosurgery was conducted using a Joule-Thomson based argon cryoprobe with the tip temperature maintained at -100°C .

Cryosurgery was conducted until the edge of the iceball overlapped the visual edge of the tumor. Toxicity (animal death) and tumor growth was monitored for a period of 30 days after treatment. The results show that CYT-6091 pre-conditioning followed by cryosurgery enables complete tumor regression without toxicity whereas significant toxicity is associated with native TNF- α pre-conditioning [84].

Figure 6. Potential for Non-invasive Imaging of Neutrophil Infiltration into Thermal Lesion (a) Leukocyte (neutrophils) infiltrate at day 3 post cryosurgery with or without TNF- α pre-conditioning. Animals were treated with cryosurgery alone, TNF- α 4 hours pre-conditioning plus cryosurgery, or NF κ B inhibitor BAY followed by TNF- α 4 hours pre-conditioning plus cryosurgery. Representative image of neutrophilic infiltrate with H&E staining was taken under high power (100 $\times$ magnification) within the inflammation zone (scale bar =100 μ m). (b) Quantification of neutrophils within each histological zone under high power field. The average number of neutrophils was measured in five representative fields in each histological layer for each sample. The bar values represent mean $\pm$ SD of three to four independent experiments for each treatment. The numbers of neutrophils per field were significantly different between the combinatorial treatment and cryosurgery alone (*, $p < 0.05$) [65]. (c) Luminol bioluminescence imaging of myeloperoxidase (MPO) activity in TRAMP-C2 tumors in vivo following cryosurgery (unpublished results).

Table 1
Nanoparticles under Clinical Evaluation that Activate or Enhance Thermal Therapy

Nanoparticle Class	Examples	Thermal Therapy Application	Company	Indications
LIPOSOMAL	ThermoDox [®]	Temperature sensitive liposomes release doxorubicin within tumors treated with radiofrequency ablation	Celsion Corporation, New York, NY, USA	Liver and breast cancer
	Myocet [™]	Liposomally encapsulated doxorubicin administered as neoadjuvant chemotherapy with regional hyperthermia	Sopherion Therapeutics, Princeton, NJ, USA	Metastatic breast cancer
METALLIC	*Aurimune [™] (CYT-6091)	Colloidal gold nanoparticle tagged with TNF preconditions solid tumors to thermal therapy	CytImmune Sciences, Rockville, MD, USA	Solid tumors
	AuroLase [®]	Gold nanoshells passively accumulate within solid tumors and are activated by near infrared laser to heat tumors	Nanospectra Biosciences, Inc., Houston, TX, USA	Head and neck cancer
MAGNETIC	NanoTherm [®]	Iron oxide particles injected directly into tumors absorb high energy radiation of external magnetic field to heat tumors	MagForce Nanotechnologies AG, Berlin, Germany	Glioblastoma multiforme, prostate cancer, esophageal cancer, pancreatic cancer

* A Phase I clinical trial has been completed with CYT-6091 as a stand-alone solid cancer therapy[43]. Pre-clinical trials have shown efficacy when combined with thermal therapies as discussed in the text.

Table 2
Thermal Therapy Devices in Clinical Use for Solid Tumors

Note that AuroLase™ and Nano-Cancer® are nanoparticle-based technologies.

Thermal Therapy	Probe	Company	Indications	Web
CRYOSURGERY	SeedNet™+	Galil Medical, Yokneam, Israel	Prostate, Kidney Cancers, Uterine Fibroids	www.galil-medical.com
	CryoCare CSTM+	Endocare, Irvine, CA, USA.	Prostate, Kidney, Lung and Liver Cancers	www.endocare.com
	Visica 2TM	Sanarus, Pleasanton, CA, USA.	Breast fibroadenomas	www.sanarus.com
	Various Systems	BryMill, Ellington, CT, USA.	Dermatologic Lesions	www.brymill.com
	Erbokryo CATM	Erbe Medical, Leeds, UK.	General Surgical Use	www.erbe-med.com
RADIOFREQUENCY	Cool-Tip® RF Ablation System	Valleylab, Boulder, CO, USA.	Tumor ablation	www.valleylab.com
	LeVeen needle electrode	Boston Scientific Corp., Natick, MA, USA.	Liver and soft tissue ablation	www.bostonscientific.com
	RITA system	AngioDynamics, Inc., Queensbury, NY, USA.	Surgical oncology, tumor (≤ 7 cm) ablation	www.angiodynamics.com
	HiTT	Berchtold, Tuttlingen, Germany.	Tumor ablation	www.berchtoldusa.com
	Prostiva® RF	Medtronic, Inc., MN, USA	Benign prostatic hyperplasia	www.medtronic.com
HIGH INTENSITY FOCUSED	Ablatherm® HIFU	EDAP TMS S.A., Vaulx-en-Velin, France.	Focused surgery, Prostate cancer tumor ablation with imaging	www.edap-tms.com
ULTRASOUND	ExAblate® 2000	InSightec-TxSonics, Dallas, TX, USA.	Tumor/fibroid ablation with concurrent MR imaging	www.insightec.com
	Sonalleve	Philips, Netherlands	Tumor/fibroid ablation with concurrent MR imaging	www.philips.com
MICROWAVE	Targis®	Urologix, Inc., Minneapolis, MN, USA.	Benign prostatic hyperplasia	www.urologix.com
	Prosatron®	Urologix, Inc., Minneapolis, MN, USA.	Benign prostatic hyperplasia treatment by TUMT®	www.urologix.com
	Microwave AZM-250	Azwell, Inc., Osaka, Japan.	Liver tumor ablation, Deep tissue and surface ablation	
	Prolieve®	Boston Scientific Corp., Natick, MA, USA	Benign prostatic hyperplasia	www.prolieve.com
LASER	INDIGO® Laser System	Indigo Medical, Inc./Johnson & Johnson, Cincinnati, Ohio, USA.	Benign prostatic hyperplasia	www.ethiconendo.com
	GreenLight PV™ Laser System	AMS, Minnetonka, MN, USA.	Benign prostatic hyperplasia	www.greenlighthps.com
	AuroLase®	Nanospectra Biosciences, Inc., Houston, TX, USA	Refractory head and neck cancer	www.nanospectra.com
MAGNETIC FLUID HYPERTHERMIA	Nano-Cancer®	MagForce Nanotechnologies AG, Berlin, Germany	Glioblastoma multiforme, prostate cancer, esophageal cancer, pancreatic cancer	www.magforce.de

Table 3
Potential Pre-conditioning Agents for Thermal Therapies

This is not intended as a comprehensive list, but only to highlight the potential of tumor microenvironment targets for pre-conditioning.

Adjuvant Class	Sub-type	Preconditioning Mechanism	Potential Agents	Reference
VASCULAR	Vascular destructive agents (VDAs)	Tumor vascular injury by direct endothelial cytotoxicity, increase junctional retraction of endothelial cells, recruitment of inflammatory infiltrate, etc.	TNF- α	
			CYT-6091*	[95]
			PEG-TNF*	[96]
			CA4-P/CA1-P	[97, 98]
			DMXAA	[99]
			ZD6126	[62]
			AVE8062	[62]
			Arsenic trioxide	[61]
	Vasoactive agents	Increase in tumor vascular permeability	Bradykinin	[100]
			Histamine	
			Serotonin	
			Prostaglandins	
			Leukotrienes	
			Complement (C3a, C4a, C5a)	
			Nitroglycerin	
	Pro-coagulant/ Vasoconstriction	Reduction in tumor perfusion by thrombus formation in tumor vessels	Thromboxane	
			Fibrinogen	[101]
			tPA inhibitor	
			Phenylephrine	
			Pentobarbital	[102]
			Metaraminol	[102]
			T β R-I inhibitor (LY364947)	[103]
IMMUNOLOGICAL	TGF- β inhibition	Increase in tumor vascular permeability	T β R-I inhibitor (LY364947)	[103]
	Cytokine	Modify tumor immune environment to activate or recruit antitumor immune cell response	TNF- α	[104]
			IFN- α	[105]
			PEG-IFN	[106]
			IL-2/Histamine	[107]
			PEG-IL-2	[108]
			IL-12	[109]
	Treg modulation	Inhibition of suppressive effects of regulatory T cells on antitumor helper and effector T cell responses	Anti-CTLA4*	[71]
	TGF- β inhibition	Inhibition of immunosuppressive effects of TGF- β	Small molecule and antibody inhibitors of TGF β RI and TGF β RII in clinical and preclinical trials	[110]
	TLR activation	Immunostimulatory molecules that activate specific TLR receptors on immune cells esp. antigen presenting cells	TLR 2,3,4,5,7,9 agonists in preclinical and clinical trials	[111]
			Imiquimod	[112]
			CpG-ODN	[113]
			LPS	[104]

Adjuvant Class	Sub-type	Preconditioning Mechanism	Potential Agents	Reference
			BCG-CWS	[114]
			OK-432	[115]
			siRNA-PEI [*]	[116]
			Anti-CD40-PLA [*]	[117]

^{*} Agents administered in nanoparticle formulations.

Table 4
Preclinical Evaluation of CYT-6091 as a Pre-conditioning Agent for Thermal Therapy

Tumor Cell Line	CYT-6091 Dose *	Animal Model	Thermal Therapy	Significant Outcomes **	Reference
LNCaP Pro 5 prostate carcinoma	200 µg/kg	Mouse hindlimb	Cryoprobe: -120°C%	25% complete remission, significant TGD#	[84]
	200 µg/kg	Mouse hindlimb	Cryoprobe: -40°C%	Significant TGD	[118]
	200 µg/kg	Mouse DSFC	Cryoprobe -120°C%	Iceball edge = tumor necrosis edge	[84]
ELT-3 uterine fibroma	80–200 µg/kg	Mouse hindlimb	Cryoprobe -120°C%	Significant TGD	[85]
SCK mammary carcinoma	125–250 µg/kg	Mouse hindlimb	Hot Water Bath: 42.5°C, 60 min	Significant TGD, enhanced tumor perfusion defect	[41]
FSaII fibrosarcoma	50–250 µg/kg	Mouse hindlimb	Hot Water Bath: 42.5–43.5°C, 60 min	Significant TGD, enhanced tumor perfusion defect	[40]
VX2 carcinoma	200 µg/kg	Rabbit kidney	Radiofrequency ablation	Enhanced tumor cell death in ablation zone	[83]
	200 µg/kg	Rabbit kidney	Cryoprobe: -100°C%	Decreased metastases	[82]

* CYT-6091 dose was administered 4 hours prior to thermal therapy.

** Compared to thermal therapy alone.

Tumor growth delay

% Lowest temperature achieved by cryoprobe.