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Combination Thermochemotherapy For The Treatment Of Non-Muscle Invasive Bladder Cancer

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Combination Thermochemotherapy for the Treatment of Non-Muscle Invasive Bladder Cancer

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COMBINATION THERMOCHEMOTHERAPY FOR THE TREATMENT OF NON-MUSCLE INVASIVE BLADDER CANCER. Ramy Goueli, Darryl Martin, Marcia Wheeler and Robert Weiss. Department of Urology, Yale University School of Medicine, New Haven, CT.

Bladder cancer is the fourth most commonly diagnosed malignancy and is the eighth leading cause of cancer death among men in the United States of America. Furthermore, it is the seventh and seventeenth most common cancer among males and females, respectively with increasing incidence among Caucasian compared with other ethnicities. Thus it is of utmost importance to develop strategies to improve our ability to early diagnose as well improve treatments by developing new drugs or develop novel treatments. We hypothesized that improving drug potency by enhancing their uptake into tumors will improve their clinical efficacy and enhance tumor killing. We investigated the efficacy of hypothermia in synergizing the effect of various chemical therapeutic agents using different cell bladder types. The combination of hyperthermia and chemotherapeutic agents showed promising results. Hyperthermia enhanced the effect of drugs in reducing the amount of drug required to decrease the number of cells by 50% (LD50). However, the effect was variable depending on cell type and the drug tested. We also showed that hyperthermia alone increased the depth of penetration and density at which nanoparticles could penetrate the bladder. Thus, combination hyperthermia and chemotherapy holds promise for the treatment of superficial bladder cancer both as an initial therapy and as a salvage therapy.

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I. INTRODUCTION

Significance

Bladder cancer is the fourth most commonly diagnosed malignancy and is the eighth leading cause of cancer death among men in the United States of America. Furthermore, it is the seventh and seventeenth most common cancer among males and females, respectively[1] with increasing incidence among Caucasian compared with other ethnicities [2, 3]. The probability for developing invasive bladder cancer in the United States is 3.81% and 1.15% for men and women, respectively [3].

Staging of Bladder carcinomas

Staging of bladder cancer from T0 to T4 is dependent on the depth of tumor penetration and the depth of tumor penetration from T0 to T4 is correlated with clinical outcome. Stage T0 denotes no evidence of primary tumor. Ta and carcinoma *in situ* (Tis) do not invade the lamina propria but rather grow along the urothelial surface [4, 5]. Once there is invasion to the lamina propria, the tumor is staged as T1, which is further divided into T1a and T1b lesions; where T1b lesions have a deeper invasion past the lamina propria and into the muscularis mucosa [6, 7]. Stage T2 lesions denote invasion into the muscularis propria, and also is subdivided into T2a where there is invasion into the inner half of the muscle layer, and T2b where there is deeper invasion into the muscle layer. When the tumor invades beyond the bladder wall, it is staged as either a T3 or T4. A T3 lesion denotes invasion into the perivesical tissue/fat, either microscopically, T3a, or macroscopically T3b[8]. T4 lesions denote that the tumor invasion extends beyond the perivesical tissue. These lesions are further stratified as T4a lesions when the tumor

invades the uterus, vagina, or prostatic stroma, and T4b lesions whe the tumor invades either the pelvic or abdominal walls [9, 10].

Approximately 90% of bladder cancers are urothelial cell carcinomas (UCC) while a small percentage are classified as squamous cell carcinomas (SCC) or adenocarcinomas [11]. In Egypt, bladder cancer is the most common malignancy among males, and is mainly attributed to *Shistosomal* infections leading to SCC, although the trend is transitioning to UCC with the increased use of anti-parasitic treatments [12]. Of the urothelial cell carcinomas, about 70-85% are superficial (Ta, T1, carcinoma *in situ*) at presentation, and are classified as non-muscle invasive bladder cancer (NMIBC) [13, 14]. For patients that present with muscle invasive bladder cancer (T2-4), outcomes are poor; and nearly half die within five years of their diagnosis [14, 15].

Risk factors

Smoking is a major risk factor for urothelial cell carcinoma of the bladder and increases the likelihood of developing the disease by four-fold as compared to individuals who never smoked. The risk is directly connected to smoking duration, quantity of cigarettes, and the age of smoking initiation [16-18]. Additional risk factors include occupational hazards such as carcinogens (aniline dyes, aromatic amines, polyaromatic amines and arsenic), pelvic radiation, cyclophosphamides, the oral antibiotic drug pioglitazone, and exposure to *Aristolochia fangchi* (a Chinese herb) [19].

Diagnosis

The most common initial presentation of bladder cancer is hematuria, either macro- or microscopic. Gross hematuria (macroscopic) has been shown to carry a 19% chance of urinary tract malignancy, while microscopic hematuria has a 5% chance of

urinary tract malignancy [20, 21]. Additional symptoms that are associated with bladder cancer include irritative voiding symptoms (dysuria, urgency, increased frequency), in the absence of an infection. Rarely, patients will present with signs of advanced disease such as, cachexia and abdominal or bone pain.

If bladder cancer is suspected, the patient should undergo a urine sample for cytology, upper urinary tract imaging, and a diagnostic cystoscopy. Urine cytology has an overall sensitivity of 30-50%. It is most sensitive (up to 90%) for patients with high grade tumors and CIS as these tumors often shed malignant cells [22]. Upper urinary tract imaging can help confirm the presence of upper tract tumors and include intravenous urography, retrograde pyelography, CT urography, and MRI studies [1]. Despite the use of urine cytology and imaging, cystoscopy continues to remain the gold standard for diagnosing bladder cancer, with a sensitivity of 94%, although it has a 60% sensitivity for diagnosing CIS [23, 24].

Transurethral resection of the bladder tumor (TURBT) is the initial diagnostic procedure for patients with bladder cancer. TURBT allows for more accurate staging, decreases or eliminates tumor burden, and helps dictate further treatment options [25]. If the tumor is found to be muscle invasive (T2 or higher) but organ confined the definitive treatment is radical cystectomy with a urinary diversion, although it may cure only 50% of patients [25, 26]. When systemic chemotherapy is indicated, for metastatic disease, there are several neoadjuvant and adjuvant options which include an MVAC regimen (Methotrexate, Vinblastine, Adriamycin, and Cisplatin), gemcitabine, or platinum based drugs. Although these agents have improved survival rates, they are also known to have severe toxic side effects. [27-29].

Treatments

A significant portion of NMIBC tumors will recur after treatment and many will display tumor progression. All patients require long-term follow-up with urine cytology and cystoscopy. The overall sensitivity of urine cytology is approximately 35% [30, 31] and cystoscopy is intrusive, uncomfortable to the patients, and costly. In addition, because of the need for monitoring the patients for an extended period of time, the cost per case for bladder cancer can be among the highest among all cancer types. Direct delivery of drugs into the bladder has proven to be an effective method to ensure maximal delivery of therapeutics to the site of the disease and to minimize systemic side effects[32]. The effectiveness of the treatment depends on the residence time of the drug inside the bladder and its binding to and penetration of the bladder wall.

The current therapies for bladder cancer, after TURBT, are intravesicular instillations of *Bacillus Calmette-Guerin (BCG)* or Mitomycin C (MMC). BCG is an attenuated mycobacterium that was originally developed as a vaccine for tuberculosis, but over the course of the past 20 years has become a standard for the treatment of NMIBC[33] . Although the mechanism of action of BCG is not fully understood, it is believed to cause the release of several cytokines (interferon-γ, interleukin-2, tumor necrosis factor) in the bladder, which ultimately trigger a non-specific immune response that results in the release of macrophages, T cells, B cells, and natural killer cells [34]. BCG is effective against NMIVC in preventing disease, and is the only drug proven to decrease disease progression. In addition, it is the only agent approved by the US Food and Drug Administration for treatment of CIS [35-37]. However, the response to BCG is unpredictable; approximately 20% of patients discontinue therapy due to side-effects,

30% of patients do not respond to BCG therapy, and more than 25% of patients have disease progression despite treatment [38-40].

Mitomycin C (MMC) is an anti-tumor antibiotic that inhibits DNA synthesis through crosslinking DNA strands [41]. MMC is a relatively large drug, 329g/mol, which greatly reduces its transurothelial absorption [42]. In terms of overall survival benefit, MMC has similar results to BCG, although, unlike BCG it has not been shown to decrease disease progression [43-45]. When MMC and BCG maintenance therapy was compared in large meta-analyses for the treatment of patients with Ta and T1 UCC, it was shown that BCG was superior at decreasing tumor recurrence in Ta and T1 UCC populations compared to MMC [35, 46, 47]. Other classes of chemotherapeutics that have been tested for the treatment of bladder cancer with some efficacy include antimetabolites (methotrexate, 5- fluorouracil), microtubule inhibitors (docetaxel, paclitaxel), and additional anti-tumor antibiotics (epirubicin, doxorubicin) [48-51].

Histone acetyl transferases (HATs) and histone deacetylases (HDACs) are known signaling enzymes that are implicated in several biochemical processes including transcription and translation and thus are involved in cellular differentiation, proliferation, and cell death. Inhibitors of HDAC (HDACis) induce transformed cell cycle arrest, terminal cell differentiation, and cell death through activation of the apoptotic pathway. HDACis also block angiogenesis [52, 53]. In fact, one HDACI (vorinostat) has been approved by the FDA for treating patients with cutaneous T-cell lymphoma, and at least 12 different HDACis have been evaluated in over 100 clinical trials involving patients with hematologic and solid tumors including lung, breast, pancreas, renal, and bladder cancers, melanoma, glioblastoma, leukemia, lymphoma, and

multiple myeloma. These inhibitors are effective in inhibiting the growth of transformed cells and are relatively less toxic for normal cells [54]. The histone deacetylase inhibitor, Belinostat (PXD101), represents a promising new anticancer therapy, which presently is under Phase III clinical trial.

Barriers to Current Therapies

The urothelium is the epithelial layer of the bladder and represents the major barrier to the permeation of drugs into the bladder wall. Thus, altering the permeability of the urothelium by minimizing trans-epithelial resistance may enhance drug delivery and efficacy[32]. The two main approaches to increase drug permeability of the urothelium include physical methods and chemical permeation enhancers. Due to the limited permeability of the bladder wall and the undesired side-effects of chemical enhancers such as dimethyl sulfoxide (DMSO) or protamine sulfate (PS), it is of interest to devote efforts to either designing carriers to deliver drugs into the bladder wall or to modulate therapy by enhancing drug attachment to the bladder wall [55, 56]. Examples of carriers under current development include liposomes, cell-penetrating peptides, thermosensitive hydrogels and PLGA nanoparticles. These have shown some positive results in the treatment of NMIBC, but more research is needed before they become clinically useful therapeutic strategies [55, 57-61]. It has been demonstrated that modulating therapy through new techniques such as electromotive drug administration, which modulates the intravesicular electric field to enhance drug penetration, is more effective than passive diffusion in delivering drugs deeper into the bladder [62-64]. An additional technique that recently has been re-visited is the addition of hyperthermia to current intravesicular drug instillations.

Hyperthermia

The addition of hyperthermia to drug therapy has been shown to improve patient outcomes in several cancers, including melanoma, head and neck cancer, breast cancer, pancreatic cancer, peritoneal cancers, ovarian, cervical and rectal cancers [65-70]. Hyperthermia has a multifactorial effect on tumor growth and whole body physiology including, independent, cytotoxic effects, vascular effects, immune effects, and thermosensitization effects[71]. The rationale of introducing hyperthermia is to make use of the difference in heat tolerance between normal and neoplastic cells; hyperthermia selectively kills the cancerous cells [72].

Therapeutic hyperthermia has a biphasic direct cytotoxic effect on exposed cells. The first phase exists between 41°-43°C, showing a reversible growth arrest on the exposed cells, with a reduction in RNA and DNA synthesis. At these temperatures, there is a cell cycle arrest at the DNA synthesis phase of the cell cycle (S) as well as at the mitosis(M) phases, which have been shown to have the greatest degree of nuclear fragility[73]. The second phase of direct cytotoxicity is at temperatures above 43°C which cause an irreversible growth arrest , with protein denaturation, impaired DNA repair, and ultimately, apoptosis[74, 75].

The effects of hyperthermia on the vasculature have been well studied. Heating the tissue to between 38°-43°C selectively targets tumor-specific vasodilation leading to increased drug delivery, direct corpuscular injury, and intravascular thrombosis related to local acidosis. Heating the tissue beyond 43°C causes endothelial cell swelling, microthrombosis, and increased vascular permeability which ultimately decreases tumor blood flow and drug delivery[76-79].

The increase in temperature, mimics fever-like conditions and causes several effects on the immune system. When the tissue is heated to 38°-43°C there is an upregulation of heat shock proteins which act as danger signals to the body to activate dendritic cells and other antigen-producing cells [80, 81]. Subsequently, the dendritic cells can recruit CD8+ cytotoxic cells which then induce an anti-tumor immune response. In addition, hyperthermia improves lymphocyte trafficking by increasing the expression of Inter-Cellular Adhesion Molecule 1(ICAM-1), Interleukin-6 and Interleukin-10, which helps increase the density of tumor-recognizing T cells in the area[82, 83]. Lastly, hyperthermia leads to a chemokine release from the tumor cells which has an effect on the innate immune system by activating natural killer cells to target these tumor cells[84-86]. At higher than fever-range temperatures, greater than 43°C, there is a down regulation of heat shock proteins and local immunosuppression[87].

When therapeutic hyperthermia (43°C) was tested on normal bladder cancer cells and five different UCC cell lines, it was shown that the effects on bladder cells was highly variable and limited [88]. Further studies investigated the effect of supra-physiological hyperthermia (40°-70°C) on three UCC cell lines and showed that the higher the cytologic grade, the lower the tolerance to heat; however, these studies lack clinical application as they used supra-therapeutic dosages of heat [89]. The synergistic effect of combining chemotherapy and hyperthermia was studied *in vitro*. In addition, van der Heijden and colleagues investigated the effect of combining hyperthermia with varying concentrations of mitomycin C, epirubicin, gemcitabine, and a bioreductive alkylating indoloquinone (E09) on four bladder cancer cell lines. In these studies, they clearly demonstrated a variable synergistic relationship between hyperthermia and drug

administration for all drugs tested across all cell lines, with the greatest synergism shown with E09 [90, 91]. A small singular animal study was performed on sheep with an hour treatment of hyperthermia (41°-43°C) and mitomycin C, and showed that this combination was safe, as there was no irreversible damage to adjacent organs nor to the bladder itself [92]. An additional preclinical study was performed to investigate the effect of temperature on urine viscosity. It was discovered that the viscosity of human urine was dependent on temperature, while being independent of specific gravity, age, gender, glycosuria, ketonuria and hematuria [93].

Several clinical studies have been performed to investigate the effects of combined hyperthermia with mitomycin C therapy [87, 94-107]. Hyperthermia was proven to increase systemic absorption of Mitomycin C, to a level up to 20ng/mL (myelosuppression occurs at 400 ng/mL), indicating that the addition of heat can increase the depth at which MMC penetrates the bladder [105]. A systematic review of the published literature showed that the combination of MMC and hyperthermia caused a 59% reduction in NMIBC recurrence when compared to MMC treatment alone [87, 97, 98]. Moreover, the effect of hyperthermia and chemotherapy may be longer lasting than current therapy, with a 3-5 year disease free rate of between 40-50% [103, 104, 108]. In addition, there has been evidence that the combination can reduce the risk of disease progression when compared to patients receiving MMC, alone [103]. However, it also was shown that combination chemotherapy plus hyperthermia increases side effects. These effects are not life threatening, they do not prevent patients from completing therapy, and resolve spontaneously after completing therapy [90, 100, 108].

Model System

It is well recognized that whole animal xenograft and orthotopic models are the gold standard for testing drug delivery to cancer cells. However, these models are cumbersome, expensive, time consuming and difficult for quantitative assessment of tumor size at various time points. Alternative models available include testing drugs on cells grown in monolayers or 3-D cultures. However, although superficial bladder cancer cells grown in monolayer are highly sensitive to cytotoxic agents, they do not simulate the resistance often seen in multilayered cell populations in vivo[109]. The benefits of using a system that combines the positive attributes of the whole animal model and the ease of manipulation of cells in culture, with minimal toxicity to other tissues or organs has been demonstrated. The organ culture model consisting of a co-cultured explanted rat bladder with bladder cancer cells, as simulation of the *in vivo* animal model was investigated [110]. The strength of this model is that it allows tumor cells to interact with transitional epithelium, extracellular matrix, and muscle of bladder [111].

II. HYPOTHESIS AND AIMS

Conflicting reports on the synergistic effect of chemotherapy and hyperthermia have been published [90, 91]. Those studies reported variable efficacies of the synergistic effect of hyperthermia with several agents such as MMC, gemcitabine, epirubicin and E09 in four bladder cancer cell lines. These studies also were limited to three cell lines (RT112, RT4, T24) of low-grade disease, with only one cell line (253J) representing high grade invasive disease. In our study, we sought to expand the bladder cancer cell lines to include both low-grade cell lines (RT4, T24) and high-grade (UM-UC-3, TCCSUP) cell lines. The UM-UC-3 cell line represents a model of high-grade invasive disease whereas the TCCSUP cells represent a model of high-grade, invasive disease with metastatic potential. Additionally, by testing the effects of the combination of MMC and hyperthermia on cell proliferation, we could compare our study with earlier studies in order to clarify the numerous inconsistencies in earlier studies that also combined MMC and hyperthermia.

Thus, the followings are the overall goals of our studies:

1. To characterize the effects of hyperthermia on bladder physiology and test if the addition of hyperthermia can act synergistically with chemotherapeutic agents. We aim to test this system using hyperthermia combined with doxorubicin, vinblastine, methotrexate, cisplatin, and belinostat to determine whether the effect of combined therapies is bladder cell line specific. Towards this goal, we will use various biochemical and molecular techniques to monitor the effects of treatments using tissue culture models. We hypothesize that hyperthermia will increase the efficacy of all chemotherapeutic agents, and be more effective in the invasive, higher grade urothelial carcinoma cell lines.

2. To investigate the effect of hyperthermia on mouse bladder urothelium and detail the histologic, gross changes since no studies have been published detailing the effect of hyperthermia, alone, on normal bladder urothelium. We hypothesize that hyperthermia primarily affects the urothelium by, reversibly, affecting the tight junctions to allow for deeper penetration of the intravesicular agent. We do not believe that the hyperthermia will have any effect on the underlying smooth muscle layer. Conversely, supra-therapeutic temperatures will cause necrosis of the urothelium with sloughing of tissue. The effect of hyperthermia on penetration of drug has been investigated previously *in vivo*, however, the authors used systemic concentration of MMC as a surrogate marker for penetrance [105].
3. To establish an ex vivo bladder cancer model that has the ability to mimic cancer growth and invasion. This system may improve the testing of combinations of hyperthermia and chemotherapy.

III. MATERIALS AND METHODS

Cell lines and culture conditions

The human urothelial cell carcinoma cell lines used in this study were RT4, T24, UM-UC-3 and TCCSUP. The cell lines were purchased from the American Type Culture Collection (ATCC), all four cell lines originated from primary bladder tumors. These cell lines varied in levels of differentiation, invasiveness and metastatic potential (**Table 1**) [112]. The RT4 cell line was originally isolated from a 63 year old male with transitional cell papilloma; it behaves *in vitro* as a low-grade superficial tumor [113, 114]. The T24 cell line was isolated from an 81 year old Caucasian female with low-grade invasive UCC. It is the most well-differentiated of the invasive UCC cell lines [115-117]. The RT4 and T24 were cultured in McCoy's 5A medium (ATCC) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin G (100 U/mL), streptomycin (100 µg/mL), and 1% L-Glutamine. The UM-UC-3 cell line was derived from a male with invasive transitional cell carcinoma. Unlike T24, it is a high-grade lesion. In addition, it is used in xenograft models because of its ability to produce tumors in athymic nude mice [118]. The TCCSUP cell line was isolated from a 67 year old female with anaplastic UCC that had metastasized to the bone. This cell line is the most undifferentiated, invasive cell line with metastatic potential [119]. The UM-UC-3 and TCCSUP cells were cultured in Dulbecco's Eagle's Minimum Essential Medium (DMEM) (ATCC) supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 µg/mL), and 1% L-glutamine. All four cell lines were grown in a monolayer on 100 mm^2 culture dishes (Corning) at 37°C in a 5% CO_2 humidified atmosphere. The cells were grown to 75-80% confluence and detached using a 0.25% trypsin EDTA mixture (Gibco).

Thermo-chemotherapy treatment

The thermo-chemotherapy treatments began by seeding all four cell lines (RT4, T24, UM-UC-3, and TCCSUP) onto 96-well microtiter plates (Falcon) at a concentration of 1.0×10^4 cells per well. The plates were placed under normal conditions for a 24 hour attachment period to allow the cells to attach to the plate. Following the 24 hours, the medium was changed and the plates were treated with increasing concentrations of various chemotherapies diluted in cell specific culture media. The chemotherapies tested were mitomycin C (Fischer Scientific) 0-1000µg/mL, doxorubicin (Sigma) 0-500µg/mL, vinblastine (MP Biomedical) 0-1000µg/mL, cisplatin (Santa Cruz) 0-2.5mM, methotrexate (MP Biomedical) (0-500µM) and belinostat (TopoTarget) 0-1mM. Subsequently, replicate plates were transported to a 5% CO_2 humidified incubator at either 37°C or 42.5°C. Following 60 minutes of treatment the chemotherapies were aspirated and the cells were washed three times in normothermic (37°C) culture specific media. The cells were then left for 24 hours in a 5% CO_2 humidified incubator at 37°C.

WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene Disulfonate) assay

Cell viability was tested using a colorimetric assay, WST-1 solution (Clontech) which is suitable for an in vitro model. The assay is based on the enzymatic cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehyrogenases present in viable cells. Twenty-four hours after treatment, the cell media was aspirated and 100uL of pre-mixed WST-1 (final dilution 1:10) was added to each well. Following the addition of WST-1 the cells were placed in a 5%CO_2 humidified incubator at 37°C for 0.5 to 4 hours. The cell viability was determined on a plate reader (Molecular Devices SpectraMax 250) by measuring the absorbance at 420-480nm. For chemotherapies where

the LD50 was achieved, three independent experiments were performed; all experiments were done in quintuplicate. Conversely, for chemotherapies that either showed no activity or the LD50 were not achieved, two independent experiments were performed, in quintuplicate, to minimize the possibility for systematic errors.

LD_{50} and cell viability calculations

To calculate the LD_{50} of the chemotherapeutic agents, the data were normalized where untreated cells were considered to have 100% cell survival. Previous literature has shown that cell killing by hyperthermia alone was minimal [88, 91]. Our own data mirrored these results (**Table 2)** thus we could confidently set untreated cells to have 100% cell survival for both temperature conditions. An ANOVA was performed for each cell line comparing the cell viability data independently at both temperatures for all dosages tested using GraphPad Prism Software (Graphpad Software, Inc.). If the ANOVA proved statistical significance for the dosages, then the data were modeled to a two phase decay equation using GraphPad Prism software (GraphPad Software, Inc.):

$$Cell\ Viability = Plateau + Spanfast \times exp^{-K_{fast}(C)} + SpanSlow \times exp^{-K_{slow}(C)}$$

A correlation coefficient was generated from the model, R^2, and was used as our measure for "goodness of fit." Using the generated equations, we were able to approximate the LD_{50} and cell viability data. To show that the two conditions showed a significant difference, statistics were performed using the GraphPad Prism Software.

Synergism calculations

The equation used to approximate the synergism between chemotherapy and hyperthermia after 60 minutes of therapy ($S_{T(60)}$) was derived and adapted from van der Heijden, Jansen et al. 2004 [90]. The equation is as follows:

$$S_{T(60)} = \frac{(dead\ cell\ fraction\ post\ hyperthermia + chemotherapy) - (dead\ cell\ fraction\ post\ chemotherapy\ alone\ )}{(dead\ cell\ fraction\ post\ chemotherapy\ alone)} \times 100\%$$

The drug concentrations used in the $S_{T(60)}$ calculation is the LD_{50} for each cell type determined under hyperthermic conditions. For each chemotherapeutic agent and cell line, synergy was tested using a two-sided unpaired t-test using the Holm-Sidak method for multiple comparisons with a confidence level of 95% and α=0.05.

Mouse bladder hyperthermic model

All mouse studies were approved by the Institutional Animal Care and Use Committee of Yale University (New Haven, CT). To test the effects of hyperthermia alone on the bladder, C57BL/6 Mice (Charles River Laboratories) were euthanized using 20% isoflurate in propylene glycol followed by cervical dislocation when mice no longer responded to toe pinch, in a method congruent with the recommendation of the Panel on Euthanasia of the American Veterinary Medical Association. Following euthanasia, the mice bladders were removed sterilely and transferred to a chemical safety hood, where the ureters and urethra were removed, and the bladder was cut into four equal pieces. After washing the explants in sterile PBS, the pieces of tissue were suspended in sterile PBS and placed into separate eppendorf tubes (GeneMate). The tissue pieces were placed

in a humidified incubator at 37 ° C, 42.5°C, or 50°C. The tissue was allowed to equilibrate to the temperature for 15 minutes and then was maintained at that temperature for either one hour, two hours, or 24 hours. The control was mouse bladder tissue fixed immediately after its removal. Following treatment the tissue was fixed in 10% neutral buffered formalin (VWR international) for 24 hours and transported in 70% Ethanol (Decon). The tissue was sectioned and H&E stained by Yale pathology tissue services (Developmental Histology Lab). Imaging was performed using a light microscope (Olympus) and images were taken using the AM Scope camera. Three independent experiments were performed and independently analyzed by members of the Weiss lab in the Department of Urology, under single-blinded conditions.

Human urothelium penetration studies

The use of human tissue was approved by the Human Investigation Committee at Yale University (protocol: 0710003157). To determine the penetration of drug under hyperthermic conditions human ureter, de-identified tissue was obtained from Yale urologic surgeons. The ureter was maintained in sterile normal saline and used within four hours of collection. The tissue was cleaned and washed in sterile normal saline, following washing the tissue, it was bisected, placed in an autoclaved 96-well dot blot chamber (Biorad) with the luminal urothelium facing upward. PLGA (*poly(lactic-co-glycolic acid))* nanoparticles fabricated by Christopher Hoimes, in the W. Mark Saltzman Laboratory in the Yale Department of Bioengineering were encapsulated with Nile Red, a red fluorescent dye. It has been shown that, PLGA is stable up to temperatures of greater than 300°C; therefore, it would remain effective at temperatures less than 50°C [120]. Functionalized nanoparticles loaded with Nile Red were suspended

in sterile PBS and added to the individual wells at either 250µg NP/well or 1mg NP/well. The study had two controls, human ureter fixed immediately following its removal, and sterile PBS added to individual wells in the dot-blot. The dot-blot chambers were incubated in humidified incubators at either 37°C or 42.5°C allowing 15 minutes to equilibrate. Following the one hour incubation, the wells were washed four times with sterile PBS to remove non-adherent nanoparticles. The tissue was cored with a biopsy punch, weighed, and fixed with 10% neutral buffered formalin (VWR international) for 24 hours and transported in 70% ethanol (Decon). The tissue was sectioned by Yale pathology tissue services (Developmental Histology Lab). Then the tissue were deparaffinized in xylene (J.T. Baker) and rehydrated in decreasing concentrations of ethanol. A nuclear stain, DAPI 0.1mg/mL (Cell Signaling), was applied at 1:100 in PBS, tissue was washed and a coverslip was applied using Prolong Gold (Invitrogen). Bladder and ureter tissue naturally fluoresce green, so no additional stains were necessary. Penetration of the nanoparticles was monitored through fluorescence microscopy (Zeiss Observer. Z1). Images were independently analyzed by members of the Weiss lab, under single-blinded conditions.

Organ culture model [111]

All rat studies were approved by the Institutional Animal Care and Use Committee of Yale University (New Haven CT). Sprague-Dawley rats were anesthetized with 90mg/kg ketamine + 10mg/kg xylazine prior to bladder removal. Bladders were removed sterilely and immediately transferred to a culture hood. Then, the ureters and urethra were removed and the bladder was bisected and cut into 4-5 mm squares. Four dots of cyanoacetate glue (Indermil) were placed equidistant into a well, and the squares

of bladder were quickly transfixed, urothelium facing upward. Fifty microliters of trypsin-EDTA (Gibco) in HBSS were added to the bladder and incubated for 15 minutes at room temperature. The trypsin mixture was gently removed and 100µl of 100% FBS (Gibco) was added and quickly removed to inactivate the residual trypsin. The bladder cancer cell line, UM-UC-3, had previously been stably transfected a PCMV DsRed-Express2 Expression vector using the Xfect^{TM} transfection agent (Clontech) resulting in cells that constitutively express red fluorescent protein. These red fluorescent UM-UC-3 cells, UM-UC-3R, were placed (10^5 to 10^6) in 100µl of Waymouth medium (ATCC) with 10% FBS containing antibiotic/antimycotic agents were pipetted directly onto the bladder surface and allowed to incubate at 37° (5% CO_2, 4 hrs.). Following the cell attachment, 4 ml of complete media was carefully added to each well, allowing it to thinly cover the bladder. Attachment and growth of cells on the bladder were monitored using fluorescence microscopy (Zeiss Observer. Z1). The media was then changed twice a week, and frequently monitored for evaporation.

IV. RESULTS

Thermo-chemotherapy

We monitored the toxic effects of hyperthermia alone (42.5°C), in bladder cancer cells (RT4, T24, UM-UC-3, TCCSUP) for one-hour. Cell survival under conditions of hyperthermia alone (42.5°C) was compared with exposure to normothermia alone (37°C). The results obtained (**Table 2**) showed that there was no significant difference in cell survival between the two groups in any of the cell lines tested. Since none of these values reached statistical significance, we assumed our two populations were non-different; therefore we could assume that every cell line exposed to temperature alone could be corrected to 100% cell survival.

Incubation of all five chemotherapeutic agents with all four bladder cancer cell lines resulted in a significant decrease in proliferation. We proved statistical significance for the decrease in cell proliferation after exposure when various dosages the of chemotherapeutic agents (mitomycin C, doxorubicin, vinblastine, methotrexate and cisplatin) independent of temperature. Subsequently, we were able to construct a two phase decay equation modeled to the data to approximate the LD_{50} for both normothermic and hyperthermic conditions; a correlation coefficient (r^2) was generated as an approximation for "goodness of fit."

*Mitomycin C (**Figure 1, Table 3**)*

The combination of hyperthermia and mitomycin C had a positive impact on all four cell lines (RT4, T24, UM-UC-3, TCCSUP), as shown (**Figure 1)**. The RT4 cell line had a calculated normothermic LD_{50} of 30.1µg/mL ($p<0.05$, $r^2=0.98$), while the calculated hyperthermic LD_{50} was 12.7µg/mL ($p<0.05$, $r^2=0.98$), which was 42% of the normothermic condition. The T24 cell line had a calculated normothermic LD_{50} of

63.7µg/mL ($p<0.05$, $r^2=0.98$) while the calculated hyperthermic LD_{50} was 51.3µg/mL ($p<0.05$, $r^2=0.97$), which was 80.6% of the normothermic dosage. The UM-UC-3 cell line had a calculated normothermic LD_{50} of 120µg/mL ($p<0.05$, $r^2=0.98$), while the calculated hyperthermic LD_{50} was 50.0µg/mL ($p<0.05$, $r^2=0.99$), which was 41.4% of the normothermic dosage. The TCCSSUP cell line had a calculated normothermic LD_{50} of 124µg/mL ($p<0.05$, $r^2=0.84$), while the calculated hyperthermic LD_{50} was 58.4µg/mL ($p<0.05$, $r^2=0.92$), which was 47.1% of the normothermic dosage. The synergistic effects of hyperthermia plus chemotherapy was calculated for all the cell lines treated with Mitomycin C (**Table 4**); they were 44.1%, 3.6%, 13.2%, and 29.2% for RT4 ($p<0.001$), T24 ($p=0.20$), UM-UC-3 ($p=0.003$), and TCCSUP ($p<0.001$) cell lines, respectively.

*Doxorubicin (**Figure 2, Table 3)***

The combination of hyperthermia and doxorubicin had a positive effect on all four cell lines (RT4, T24, UM-UC-3, TCCSUP), as shown (**Figure 2).** The RT4 cell line had a calculated normothermic LD_{50} of 6.28µg/mL ($p<0.05$, $r^2=0.95$), while the calculated hyperthermic LD_{50} was 5.52µg/mL ($p<0.05$, $r^2=0.99$), which was 88% of the normothermic dosage. The T24 cell line had a calculated normothermic LD_{50} of 48.2µg/mL ($p<0.05$, $r^2=0.94$) while the calculated hyperthermic LD_{50} was 8.51µg/mL ($p<0.05$, $r^2=0.88$), which was 17.7% of the normothermic dosage. The UM-UC-3 cell line had a calculated normothermic LD_{50} of 214µg/mL ($p<0.05$, $r^2=0.92$), while the calculated hyperthermic LD_{50} was 57.3µg/mL ($p<0.05$, $r^2=0.92$), which was 26.7% of the normothermic dosage. The TCCSSUP cell line had a calculated normothermic LD_{50} of 8.62µg/mL ($p<0.05$, $r^2=0.93$), while the calculated hyperthermic LD_{50} was 6.69µg/mL ($p<0.05$, $r^2=0.98$), which was 77.6% of the normothermic dosage. The synergistic effects

of hyperthermia plus chemotherapy was calculated for all the cell lines treated with Doxorubicin(**Table 4**); they were 11.6%, 66.2%, 23.2%, and 49.6% for RT4 ($p=0.26$), T24 ($p=0.003$), UM-UC-3 ($p=0.001$), and TCCSUP ($p=0.002$) cell lines, respectively.

*Vinblastine (**Figure 3, Table 3**)*

The combination of hyperthermia and vinblastine had a positive effect on all four cell lines (RT4, T24, UM-UC-3, TCCSUP), as shown (**Figure 3).** The RT4 cell line had a calculated normothermic LD_{50} of 476µg/mL ($p<0.05$, $r^2=0.96$), while the calculated hyperthermic LD_{50} was 421µg/mL ($p<0.05$, $r^2=0.92$), which was 88.4% of the normothermic dosage. The T24 cell line had a calculated normothermic LD_{50} of 376µg/mL ($p<0.05$, $r^2=0.94$) while the calculated hyperthermic LD_{50} was 176µg/mL ($p<0.05$, $r^2=0.97$), which was 46.7% of the normothermic dosage. The UM-UC-3 cell line had a calculated normothermic LD_{50} of 915µg/mL ($p<0.05$, $r^2=0.84$), while the calculated hyperthermic LD_{50} was 403µg/mL ($p<0.05$, $r^2=0.98$), which was 44% of the normothermic dosage. The TCCSSUP cell line had a calculated normothermic LD_{50} of 553µg/mL ($p<0.05$, $r^2=0.94$), while the calculated hyperthermic LD_{50} was 198µg/mL ($p<0.05$, $r^2=0.98$), which was 35.8% of the normothermic dosage. The synergistic effects of hyperthermia plus chemotherapy was calculated for all the cell lines treated with Doxorubicin(**Table 4**); they were 21.9%, 19.1%, 86.8%, and 11.4% for RT4 ($p<0.001$), T24 ($p=0.003$), UM-UC-3 ($p<0.001$), and TCCSUP ($p<0.001$) cell lines, respectively.

*Methotrexate (**Figure 4, Table 3**)*

The combination of hyperthermia and methotrexate was performed for all four cell lines (RT4, T24, UM-UC-3, and TCCSUP), but only the UM-UC-3 showed a positive effect (**Figure 4**). The UM-UC-3 cell line had a calculated normothermic LD_{50}

of 309μM ($p<0.05$, $r^2=0.94$), while the calculated hyperthermic LD_{50} was 270μM ($p<0.05$, $r^2=0.93$), which was 87.4% of the normothermic dosage. At 0-500 μM of methotrexate, there was no effect on the RT4 cell line, independent of temperature (data not shown). Although there was a slight decrease in cell proliferation among the T24 and TCCSUP cells at hyperthermic conditions compared to normothermic conditions, the LD_{50} was not reached for either of these cell lines (data not shown). The synergistic effect of hyperthermia plus chemotherapy was only calculated for UM-UC-3, as this was the only cell line to reach the LD_{50}, 30.9% ($p=0.04$) (**Table 4**).

*Cisplatin (**Figure 5, Table 3**)*

The combination of hyperthermia and cisplatin was performed for all four cell lines (RT4, T24, UM-UC-3, and TCCSUP), but only the UM-UC-3 showed a positive effect (**Figure 4**). The UM-UC-3 cell line had a calculated normothermic LD_{50} of 576μM ($p<0.05$, $r^2=0.91$), while the calculated hyperthermic LD_{50} was 80.8μM ($p<0.05$, $r^2=0.94$), which was 14.0% of the normothermic dosage. The methotrexate concentration used ranged between 0-1000μM; this agent had no effect on the RT4 cell line, independent of temperature condition (data not shown). Although there was a substantial decrease in cell proliferation among the T24 and TCCSUP cells, the LD_{50} was not reached for either of these cell lines, additionally, there was no statistical difference between the two conditions (data not shown). The synergistic effect of hyperthermia plus chemotherapy was only calculated for UM-UC-3, as this was the only cell line to reach the LD_{50}, it was 14.0% ($p<0.001$) (**Table 4**).

Belinostat

The combination of hyperthermia and belinostat was performed for all four cell lines (RT4, T24, UM-UC-3, and TCC SUP), with none of the cell lines showing

statistical difference between hyperthermic and normothermic conditions. At 0-1mM of belinostat there was decreased cell proliferation at the highest concentrations in the T24, UM-UC-3, and TCCSUP cell lines, the LD_{50} was never reached. The RT4 cell line had no decrease in proliferation, even at the highest concentration (data not shown).

Mouse bladder hyperthermic model

The effect of temperature alone was investigated using mouse bladders placed at various temperatures (37°C-50°C) for various amounts of time (1 hour-24 hours)(**Figure 6)**. All samples were paired, from the same animal, across the three temperature conditions to minimize subject variability. The control (**Figure 6-A)** was fixed immediately after its removal, while the experimental conditions, 37°C (**Figure 6-B**), 42.5°C (**Figure 6-C**), and 50°C (**Figure 6-D**), were exposed for one hour at these temperatures before fixation. The control (**Figure 6-A**) and 37°C (**Figure 6-B**) both represent normal, healthy urothelium. In both images, the three layers of the bladder are clearly differentiated and both have a thick, well organized urothelium, **Um**. The urothelium is the location of the dome cells that maintain the impermeability of the epithelium; the cells in this layer are large, ovoid with round, uniform nuclei and evident eosinophilic cytoplasm. The normal scalloped appearance of the urothelium is maintained in these tissues. The lamina propria, **LP**, is seen in both the images as loose collagenous material with some small blood vessels providing oxygen and nutrients to the urothelium. The detrusor muscle, **DM,** is also clearly visualized in the images; the detrusor is the smooth muscle that allows contraction of the bladder. In the images, the detrusor muscle contains mononucleated cells. These cells can be seen as elongated and tapering, with

homogenous eosinophilic cytoplasm. When the tissue is exposed to therapeutic levels of hyperthermia (42.5°C) (**Figure 6-C**) there is increased disorganization of the urothelium with some evidence of pyknosis of nuclei. There is apparent exudations and vacuolization of the lamina propria, with some pyknosis visualized. The detrusor muscle is well maintained at this temperature. At supra-therapeutic levels of hyperthermia (50°C) (**Figure 6-D**), there is increased disorganization of urothelium, with apparent karyorrhexis, karyolysis, extensive cell membrane rupture and necrosis of the umbrella cells. At this temperature, there is increased exudation of the lamina propria with sloughing of the urothelium from the underlying detrusor muscle. Similar to the findings with therapeutic hyperthermia, the detrusor muscle is well maintained at hyperthermic conditions.

Depth of penetration

Human ureter was obtained and tested with nanoparticles which contained Nile Red dye to test for depth of penetration. The ureter was divided into two pieces and placed in the dot blot apparatus. The luminal side of the tissue was exposed to the fluorescent nanoparticles. These two pieces of ureter were exposed to either hyperthermic (42.5°C) or normothermic (37°C) conditions for one hour before fixation. The slides were stained with DAPI, a nuclear marker, and the slides were examined using fluorescence microscopy (**Figure 7**). The superficial images taken from the normothermic condition (**Figure 7-A**) show the Nile red particles concentrated along the urothelium, **UM**, with penetration to the Lamina Propria, **LP**. The deep images taken from the normothermic condition (**Figure 7-B**) show a slight penetration of the nanoparticles into the lamina propria. The superficial images taken from the hyperthermic

condition (**Figure 7-C**) show the nanoparticles along the urothelium with increased penetration of the nanoparticles into the lamina propria. The deep images of the tissue taken from the hyperthermic condition (**Figure 7-D**) were examined and showed a deeper penetration of the nanoparticles through the lamina propria onto the border of the detrusor muscle, **DM**. In addition to deeper penetration, the nanoparticles were present at a higher density when compared to normothermic conditions.

Organ Culture

Rat bladders were removed from Sprague-Dawley rats and seeded with UM-UC-3R cells, which were grown for one month, with frequent examinations using light and fluorescence microscopy (**Figure 8**). On day 21, light microscopy of the rat bladders (**Figure 8-A**) showed cell growth along the luminal side of the tissue. When the tissue was examined using fluorescence microscopy (**Figure 8-B**), these cells showed red fluorescence, indicating that these cells were the seeded UM-UC-3R cells. This same tissue was re-examined on day 28; light microscopy (**Figure 8-C**) showed continued cell growth along the luminal side of the tissue. Again this tissue was examined using fluorescence microscopy (**Figure 8-D**), again showing the red fluorescence of the transfected UM-UC-3R cell line.

V. DISCUSSION

Our data suggest that the effect of therapeutic hyperthermia alone does not have deleterious effect on cell viability since there was no significant change in cell proliferation among the four cancer cell lines tested (RT4, T24, UM-UC-3, TCCSUP).

Our studies with combination therapy using hyperthermia and different therapeutic agents showed a variable response depending on the cell type and the type of agent used. When we used a combination of hyperthermia and mitomycin C, we showed synergism for all the cell lines tested with the exception of the T24 cell line. Two previous studies have shown synergy with mitomycin C and therapeutic hyperthermia, but it was difficult to compare with our data as there are many inconsistencies among the two published studies [90, 91]. The authors included three cell lines (RT112, RT4, T24) of low-grade disease and one cell line (253J) representing high-grade invasive disease. For example, there was a three order of magnitude difference between the LD50 of mitomycin C for all the cell lines despite being tested using similar scientific method and cell lines. Additionally, neither of the published studies performed any statistical analyses, thus it was not possible to determine if the reported results were statistically significant. As our studies use two cell lines (RT4 and T24) that overlapped with their work, we were able to compare the two sets of results. Our data with RT4 cell line confirm their conclusions that there was synergy in combination therapy. In our studies the RT4 cell line showed the greatest synergy of the four cancer cell lines. Our data conflicted with their results for the T24 cell lines since we did not see synergy with combination therapy. Furthermore, we showed synergy between MMC and hyperthermia with two higher grade invasive cancer cell lines. The largest MMC dose decrease

occurred in the UM-UC-3 cell line, with a dosage difference of 59% to achieve the LD50 for the combination therapy compared to chemotherapy alone.

Our results with doxorubicin, an anthracycline antibiotic showed synergism for three of the four cell lines tested, but did not show synergism for the RT4 cell line. One previous study examined epirubicin, another anthracycline antibiotic, and showed synergy among all the cell lines tested [91]. In fact, epirubicin showed the greatest levels of synergy compared to all the drugs tested, in particular in the T24 cell lines, although there are a number of inconsistencies in the paper. Our data showed synergy between the T24, UM-UC-3, and TCCSUP cell lines, with the highest synergy occurring with the T24 cell line at 66.2% compared to their value of 1,823%. Unfortunately, like the MMC data, the authors, did not include statistical analyses and thus it was not possible to compare with our data [91]. The T24 cell line showed the largest change in doxorubicin dose at 82% to achieve the LD50 under combined therapy compared to chemotherapy alone.

Our results with vinblastine, a microtubule inhibitor showed that it is the only drug to display a statistically significant synergism for all the cell lines tested. No previous studies have been published examining the combination of hyperthermia with vinblastine for bladder cancer cell lines. The UM-UC-3 cell line showed the highest synergy of all the cell lines tested at 87%. The TCCSUP cell line showed the largest change, 64%, in vinblastine dose to achieve the LD50 in combined therapy compared to chemotherapy alone.

Our results with cisplatin and methotrexate only yielded positive effects on the UM-UC-3 cells. The UM-UC-3 cells had a modest change in methotrexate dosage required to achieve the LD50, while cisplatin had the largest dosage difference to achieve

the LD50 when comparing combination therapy to chemotherapy alone. Both chemotherapeutic agents have synergy with hyperthermic conditions, methotrexate more so than cisplatin. Our study is the only one to address the effect of these two chemotherapeutic agents, showing their specificity for one cell line, and thus it is unlikely that these would have wide spread application under combination therapy.

The HDAC inhibitor, belinostat, did not show any statistical difference between combination therapy and chemotherapy alone. Prior studies in the Weiss lab showed that belinostat had an LD50 of approximately 2.5uM after 72 hours of treatment (Martin et al., 2013 submitted). After 24 hour incubation with belinostat, proliferation decreases were much smaller than after 72 hours. In the current study, the LD50 was not achieved for any of the cell lines, likely due to the fact that we only exposed the cells for one hour. The difference in result suggests that either the cells need longer than a one hour exposure for belinostat's effect, or that the cells should be grown for longer than 24 hours for belinostat's effect to be observed. Alternatively, the lack of effect may be due to the instability of the drug at increased temperatures that may alter its chemotherapeutic effect.

In these studies, we looked exclusively at the increased cytotoxic effects with hyperthermia. We found that the three most efficacious drugs tested were MMC, doxorubicin, and vinblastine. These three agents are well known to be effective inhibitors of cell replication machinery. For example, MMC is a potent DNA crosslinker, doxorubicin is an inhibitor of topoisomerase II and generates free radicals, and vinblastine is a potent inhibitor of microtubule assembly, a necessary step in cellular replication. It is unclear why cisplatin would not have the same efficacy as MMC, as they

share a similar mechanism of DNA crosslinking, but it is possible that the cells could have been resistant. It is unlikely that the difference lies in the size of the molecule since cisplatin with a molecular weight of 300.5g/mol is smaller than MMC (g/mol). Conversely, the lower effect of methotrexate may be due to its mechanism of action where it acts as a folate analog to inhibit DNA and protein synthesis, processes that likely take longer than 24 hours to show its complete effect.

Of the cancer cell lines tested, UM-UC-3 was the most chemo-sensitive and overall showed the greatest synergy between hyperthermia and chemotherapy. The T24 cell line was the most chemo-resistant of the cell lines tested, although it showed the largest synergy when tested with doxorubicin. There was no connection between sensitivity to combination therapy and mutations in either tumor protein 53 (TP53) or the retinoblastoma (Rb) genes.

There are many limitations to our studies. Our limited growth time after the treatment dose may have altered our ability to notice a statistical difference in the chemotherapeutic agents tested. In particular, the HDAC inhibitor, methotrexate and cisplatin could have shown an effect if tested after 72 hours of growth. Additionally, these studies were limited as they looked exclusively at the modulation of cytotoxic or cytostatic effects of the drugs tested under combination therapy, which does not allow us to see the effects under physiologic conditions. It is possible that the treatments would only be available to the superficial cells of the tumor, with no penetration into the bladder thus hindering the efficiency of any drug tested. We also could have tested for the effect of these drugs in combination with hyperthermia on parameters including apoptosis, cell viability, and cell replication. These could be done using markers for apoptosis such as

caspases 3 and 7 (executioner proteases); DNA replication could be measured using deoxyuridine incorporation; and cell viability could be measured using ATP levels. These markers would further delineate the effect of these various drugs on cells tested.

We strived to be consistent with current therapeutic protocols, but it would be interesting to design a model where we could have our initial induction period, then have maintenance therapy to see if these cells become increasingly resistant or sensitive to combination therapy. In addition to modulating the experimental design, our current tissue culture model system does not allow us to see how the combination therapy would affect a tumor grown three-dimensionally. We designed the organ culture model as a possible way to test the efficacy of combination therapy; unfortunately, the model did not prove to be a resilient model of bladder cancer. Therefore, a xenograft or orthotopic mouse model would validate our data, *in vivo*. Lastly, it would be interesting to see how the bladder cancer cell lines would react to combination hyperthermia and BCG. It is likely that BCG would have no effect on the cell growth, as the postulated mechanism of BCG relies mainly on the immune response induced from the treatment.

Our organ studies revealed the effects of therapeutic (43°C) and supra-therapeutic hyperthermia (50°C) on normal animal tissue. With increasing temperature there was increased disorganization, necrosis and apoptosis of the urothelium. Additionally, there was increased vacuolization of the lamina propria, with evidence of urothelial sloughing at supra-therapeutic hyperthermia. This study allowed us to identify end organ changes. The main limitation to this study was that these tissues were removed from the animal, thus limiting our ability to observe the vascular or immune responses to hyperthermia. Additionally, by conducting this study *ex vivo*, we were unable to observe if there is

reversibility of the reaction following the termination of hyperthermia. In the future, it is possible to use fluorescent stains against the cellular junction proteins to identify the exact effects of hyperthermia on the urothelium. It is possible that there are changes to either the gap junctions, tight junctions, or adherens junctions that create the visualized changes and allow for the increased depth of penetration of the nanoparticles.

There have been numerous published articles detailing the depth of penetration of drugs delivered in the bladder [61], but none of these studies showed the effects of hyperthermia on this phenomenon. Our studies revealed that hyperthermia alone increased the depth of penetration and density at which the nanoparticles could penetrate into the bladder. Although the use of nanoparticles represents a good model system it does not mimic the chemotherapeutic agents in their size or charge. In studying heated intraperitoneal chemotherapy, many investigators used platinum based chemotherapies as they can be easily visualized using x-ray fluorescence microscopy [121]. Therefore using cisplatin as our chemotherapeutic agent may allow for clearer penetration studies. This study would also benefit from the use of cellular stains to determine the possible effects of hyperthermia on the bladder urothelium.

In conclusion, combination hyperthermia and chemotherapy holds a lot of promise for the treatment of superficial bladder cancer as an initial therapy and as a salvage therapy. The bladder is an excellent model to study the effects of increasing temperature on animal physiology, not only in drug delivery, but also to study the biochemical and molecular mechanisms of hyperthermia. With the increased usage of intravesicular thermochemotherapy there will be an increased need to understand the mechanisms resulting in survival benefits, to best optimize this novel therapy.

VI. TABLES AND FIGURES

TABLE 1. RELATION OF THE ORIGIN AND SUMMARY OF THE MOLECULAR CHARACTERISTICS OF THE BLADDER CANCER CELLS UNDER STUDY.

	RT4	T24	UM-UC-3	TCCSUP
Histopathology	Papillary Low-grade TCC	Invasive TCC	Invasive High-grade TCC	Invasive-metastatic High-grade TCC
TP53* mutation	Wild type	Exon 5	Exon 4	Exon 10
RB mutation**	No	No	No	No
Total RB Immunohistochemistry	10-20%	90%	70%	Negative

*TP53 IS TUMOR PROTEIN 53 **RB IS RETINOBLASTOMA GENE
TABLE ADAPTED FROM SANCHEZ-CARBAYO ET AL., 2002 [112]

TABLE 2: INFLUENCE OF HYPERTHERMIA, ALONE, ON CELL SURVIVAL

Bladder Cancer Cell Line	% Cell Survival at 42.5°C (±SD)*	Significance (p-value)
RT4	92.5%±4.3%	0.47
T24	92.5%±3.9%	0.63
UM-UC-3	91.2%±5.0%	0.23
TCC	90.7%±3.6%	0.37

*CELL SURVIVAL AT 37°C WAS ASSUMED TO BE 100% FOR ALL CELL LINES. PLATE DENSITY WAS DETERMINED USING IMAGE J (NIH)

TABLE 3: LD50 OF CHEMOTHERAPEUTIC AGENTS WITH AND WITHOUT HYPERTHERMIA

Treatment	LD_{50} RT4	LD_{50} T24	LD_{50} UM-UC-3	LD_{50} TCC
Mitomycin C				
37°C (ug/mL)	30.1	63.7	120	124
42.5°C (ug/mL)	12.7	51.3	50.0	58.4
% Δ LD_{50}	58%	19%	59%	53%
Doxorubicin				
37°C (ug/mL)	6.28	48.2	214	8.62
42.5°C (ug/mL)	5.52	8.51	57.3	6.69
% Δ LD_{50}*	12%	82%	73%	22%
Vinblastine				
37°C (ug/mL)	476	376	915	553
42.5°C (ug/mL)	421	176	403	198
% Δ LD_{50}*	12%	53%	56%	64%
Methotrexate				
37°C (uM)			309	
42.5°C (uM)			270	
% Δ LD_{50}*			12.6%	
Cisplatin				
37°C (uM)			576	
42.5°C (uM)			80.8	
% Δ LD_{50}*			86.0%	

*Δ LD_{50} IS A CALCULATION OF THE DIFFERENCE BETWEEN THE LD_{50} OF THE CHEMOTHERAPY WITH AND WITHOUT HYPERTHERMIA; IT WAS CALCULATED BY THE FOLLOWING EQUATION: $\boldsymbol{\Delta\, LD_{50} = 100 - (\frac{LD_{50\,(42.5°C)}}{LD_{50\,(37°C)}} \times 100)}$. [91]

TABLE 4: SYNERGISTIC EFFECT (IN PERCENTAGE) OF ONE HOUR HYPERTHERMIA* COMBINED WITH CHEMOTHERAPY ON THE CELL KILLING OF FOUR HUMAN BLADDER CANCER CELL LINES, COMPARED TO CELLS TREATED FOR ONE HOUR AT NORMOTHERMIA* WITH CHEMOTHERAPY

Treatment	$\%S_{LD50}$ RT4	$\%S_{LD50}$ T24	$\%S_{LD50}$ UM-UC-3	$\%S_{LD50}$ TCCSUP
Mitomycin C	44.1%	3.6%$^{+}$	13.2%	29.2%
Doxorubicin	11.6%$^{+}$	66.2%	23.2%	49.6%
Vinblastine	21.9%	19.1%	86.8%	11.4%
Methotrexate			30.9%	
Cisplatin			14.0%	

*HYPERTHERMIA=42.5° **NORMOTHERMIA=37°C $^{+}$NOT SIGNIFICANT

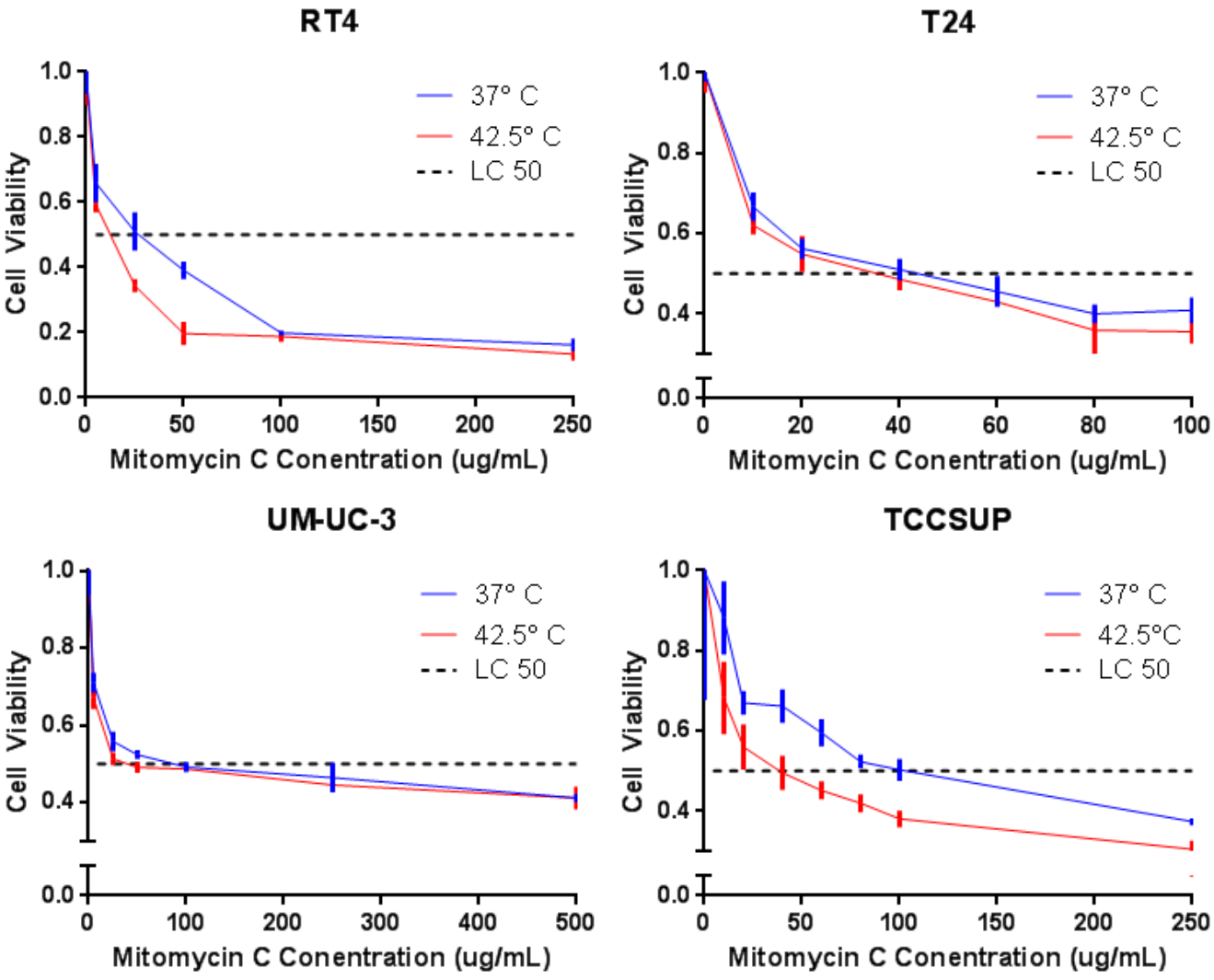

FIGURE 1: Effect of combined 1-hour hyperthermia with mitomycin c (red line) on the growth of four bladder cancer cell lines compared with normothermia and mitomycin c (blue line). Dashed line denotes LD_{50} axis.

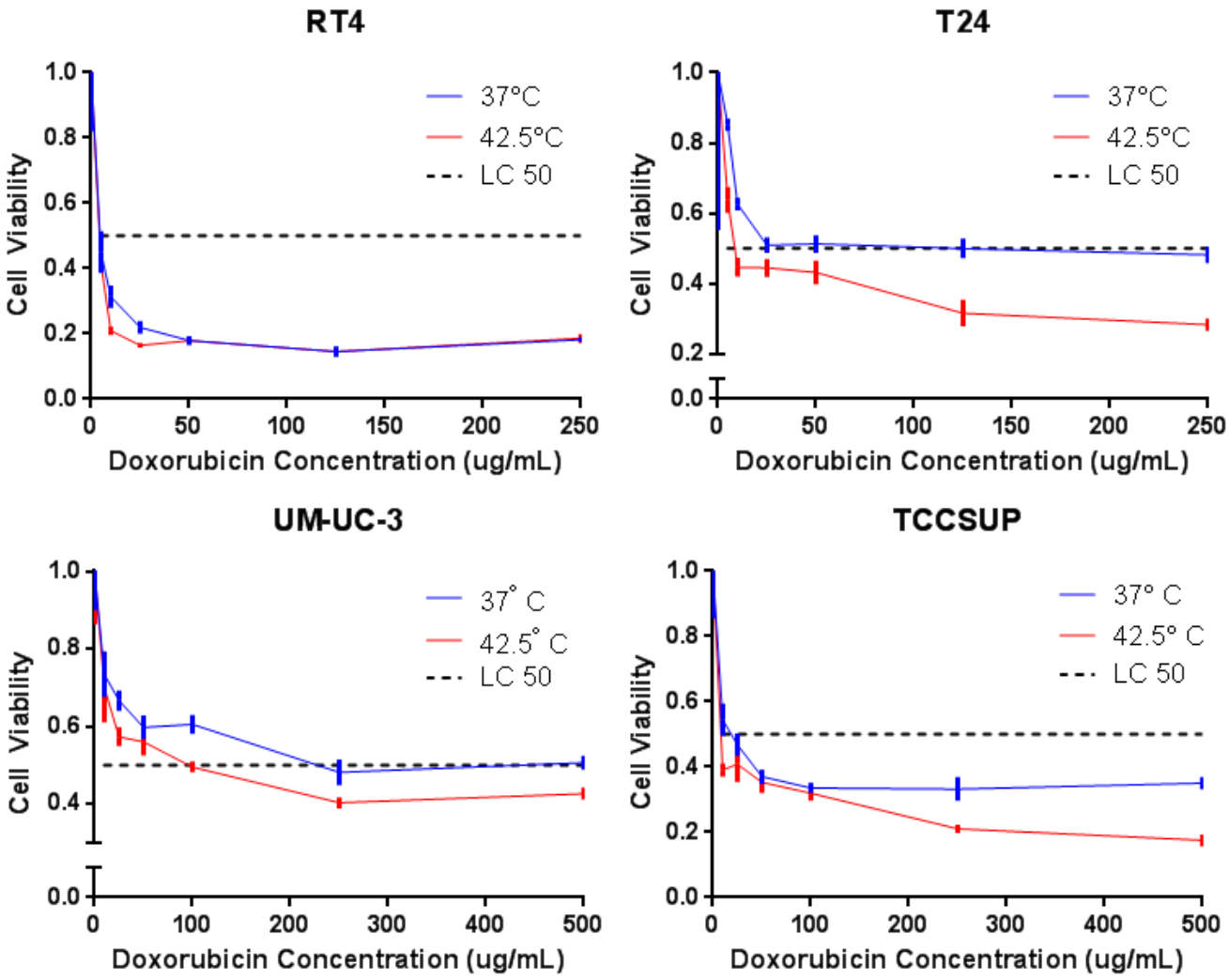

FIGURE 2: Effect of combined 1-hour hyperthermia with doxorubicin (red line) on the growth of four bladder cancer cell lines compared with normothermia and doxorubicin (blue line). Dashed line denotes LD_{50} axis.

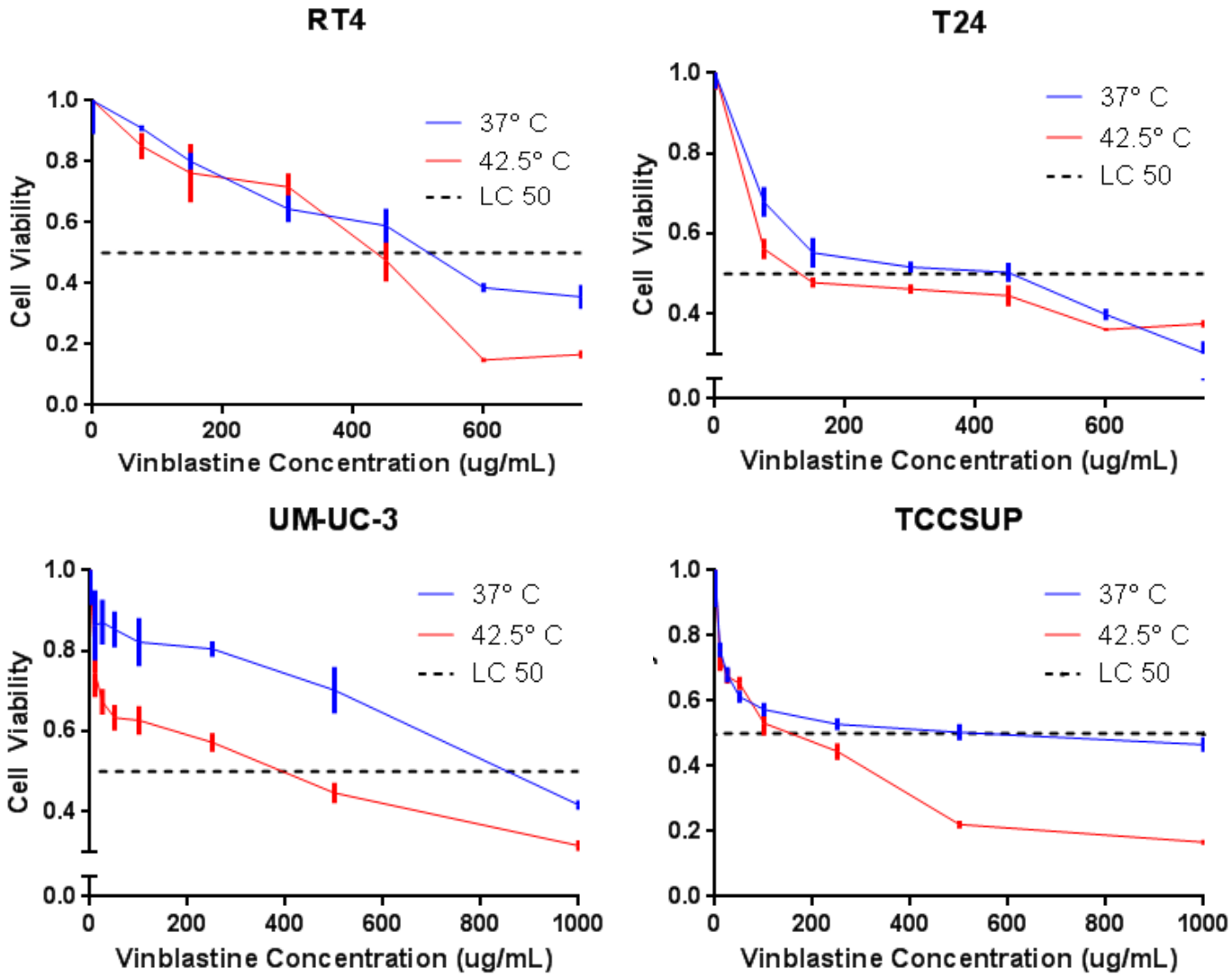

FIGURE 3: Effect of combined 1-hour hyperthermia with vinblastine (red line) on the growth of four bladder cancer cell lines compared with normothermia and vinblastine (blue line). Dashed line denotes LD_{50} axis.

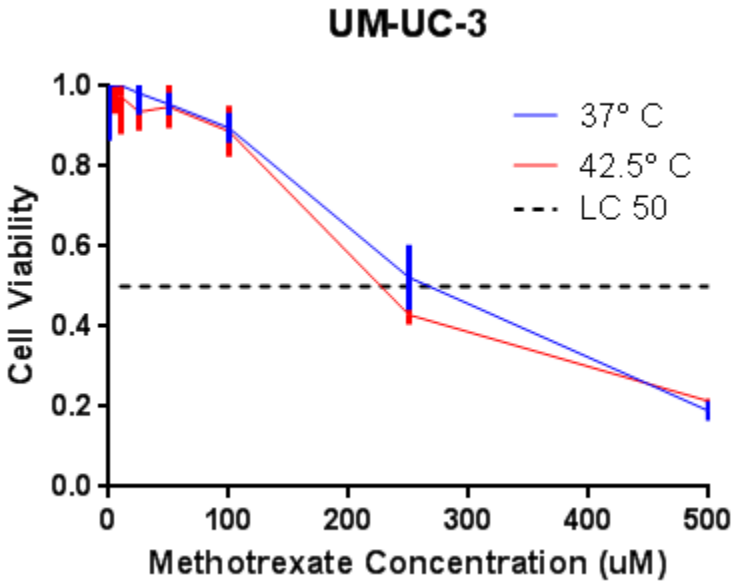

FIGURE 4: Effect of combined 1-hour hyperthermia with methotrexate (red line) on the growth of UM-UC-3 bladder cancer cell line compared with normothermia and methotrexate (blue line). Dashed line denotes LD_{50} axis.

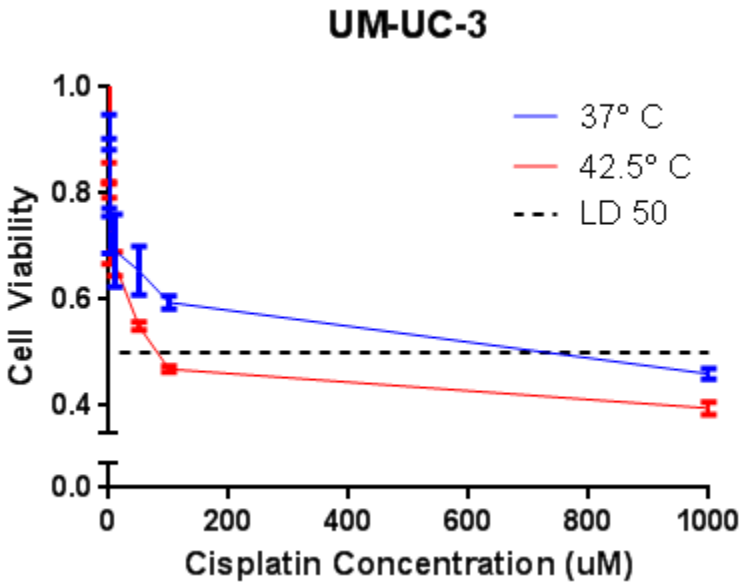

FIGURE 5: Effect of combined 1-hour hyperthermia with cisplatin (red line) on the growth of UM-UC-3 bladder cancer cell line compared with normothermia and cisplatin (blue line). Dashed line denotes LD_{50} axis.

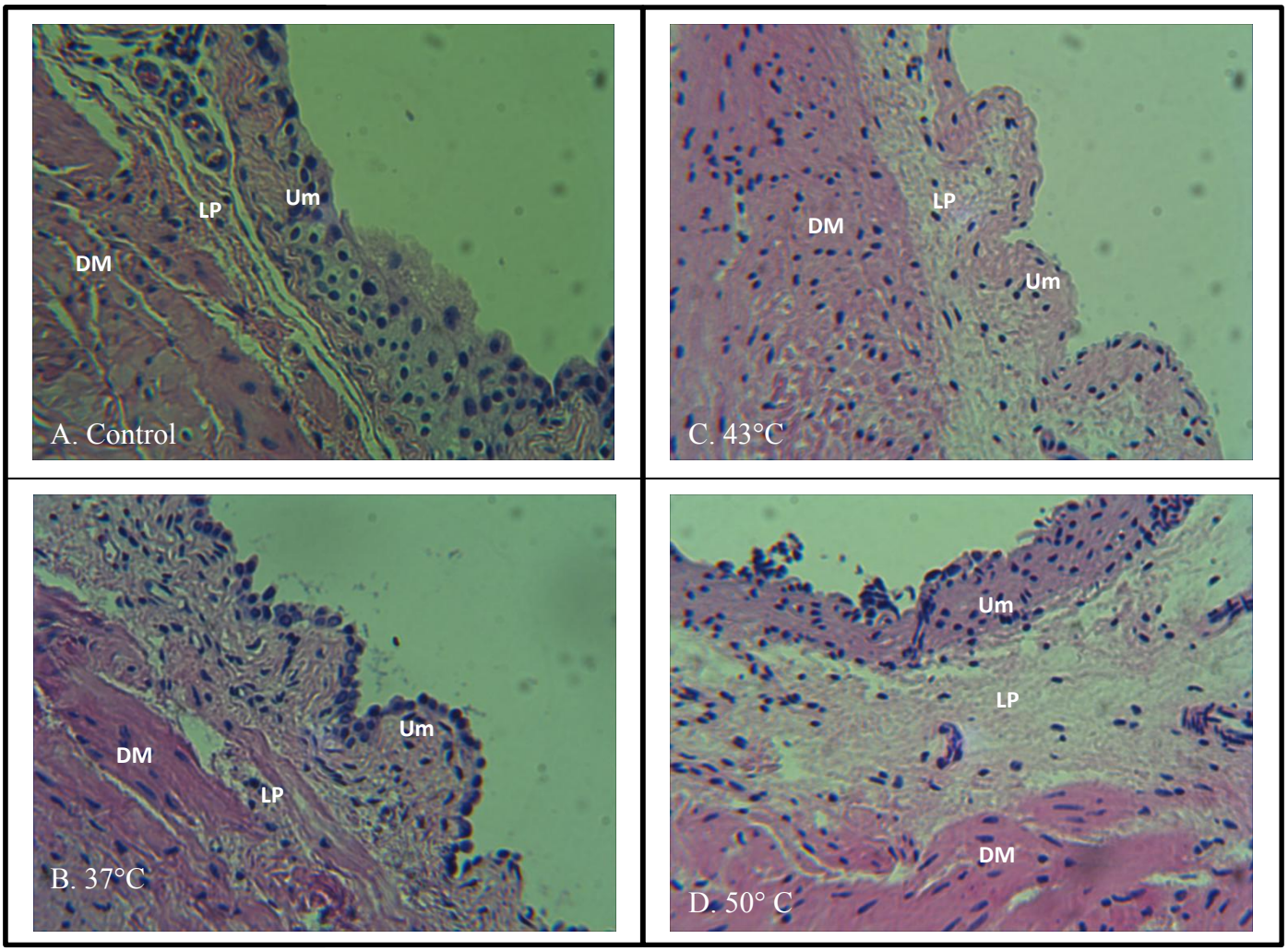

Figure 6: Rat bladders exposed to varying temperatures conditions for one hour. UM is the urothelium; LP is the lamina propria; and DM is the detrusor muscle

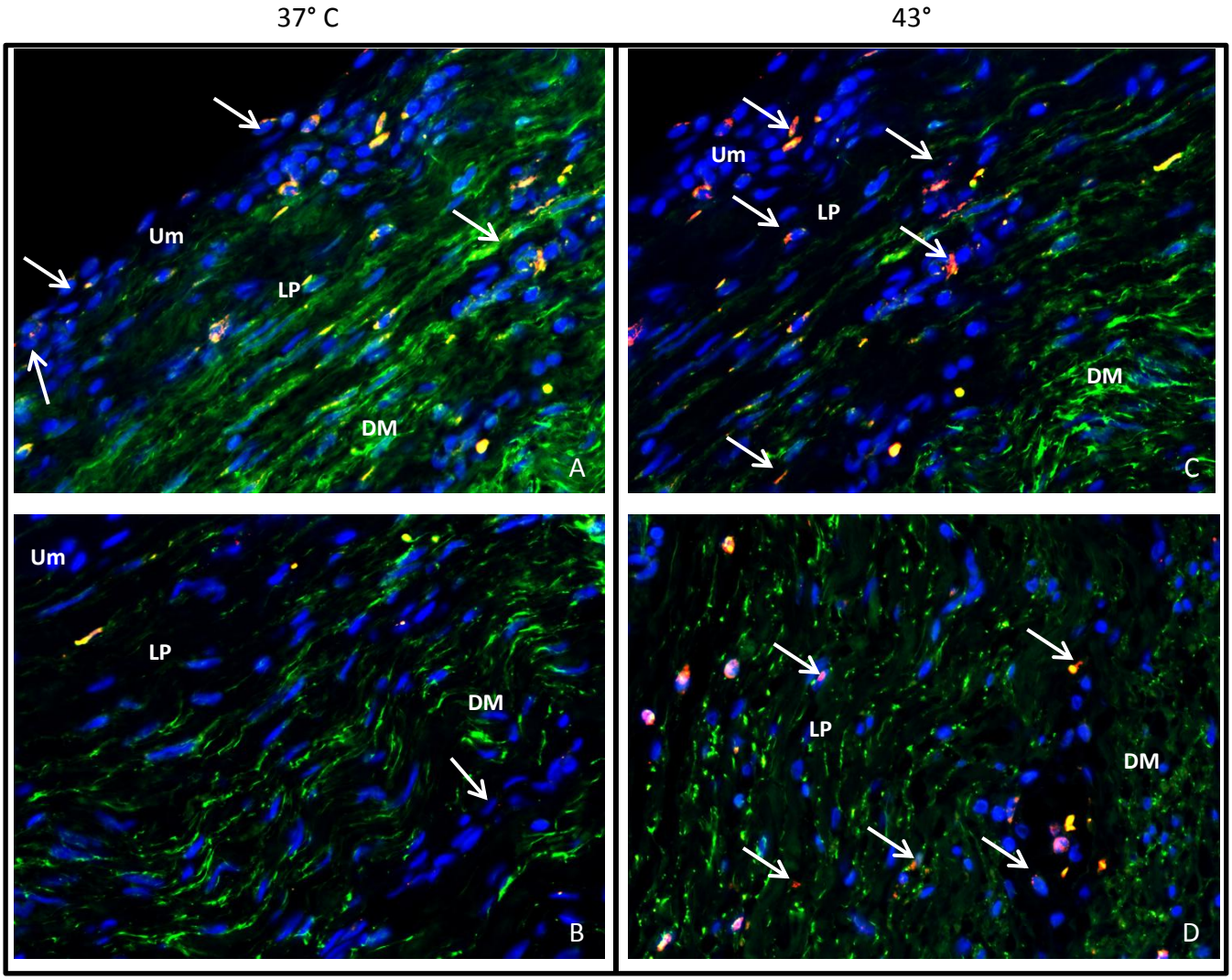

FIGURE 7: Depth of penetration experiments with human ureter exposed either to normothermic or hyperthermic conditions with Nile red nanoparticles administered on the urothelial surface for one hour. UM is the urothelium; LP is the lamina propria; and DM is the detrusor muscle. The bladder naturally fluoresces green; the blue fluorescence is the DAPI nuclear stain; the arrows indicate the location of the Nile red nanoparticles.

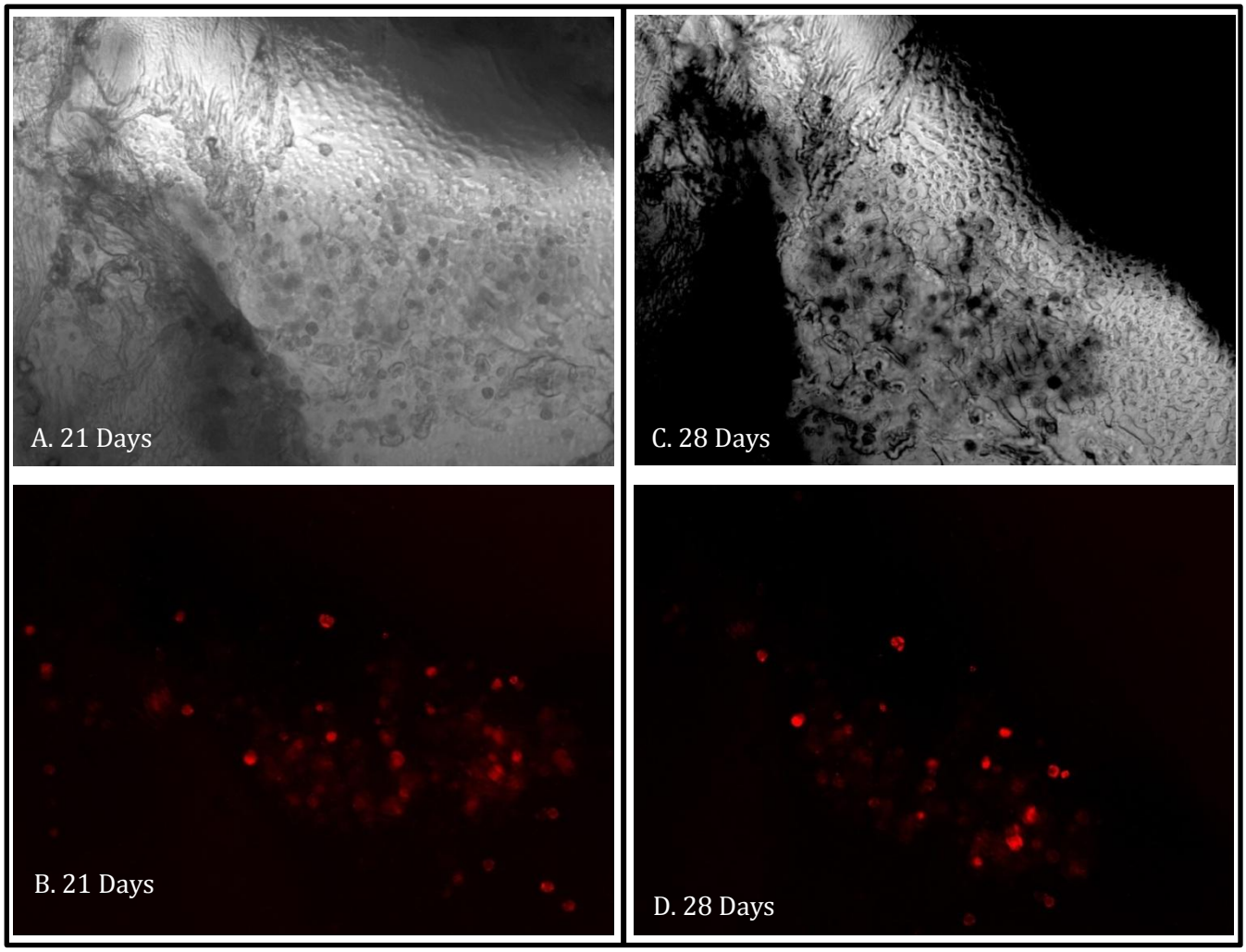

FIGURE 8: Rat bladder organ culture ex-vivo, Sprague-Dawley rats were removed and seeded with UM-UC-3 cell lines transfected with red fluorescent proteins. The tissue was allowed to grow in a 5% CO_2 humidified incubator at 37°C. The tissue was frequently examined using light and fluorescence microscopy.

VII. REFERENCES

1. Kakehi, Y., et al., *Bladder Cancer Working Group report.* Jpn J Clin Oncol, 2010. **40 Suppl 1**: p. i57-64.
2. Edwards, B.K., et al., *Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates.* Cancer, 2010. **116**(3): p. 544-73.
3. Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2013.* CA Cancer J Clin, 2013. **63**(1): p. 11-30.
4. Jones, T.D., et al., *Urothelial carcinoma with an inverted growth pattern can be distinguished from inverted papilloma by fluorescence in situ hybridization, immunohistochemistry, and morphologic analysis.* Am J Surg Pathol, 2007. **31**(12): p. 1861-7.
5. Sung, M.T., et al., *Natural history of urothelial inverted papilloma.* Cancer, 2006. **107**(11): p. 2622-7.
6. Lopez-Beltran, A. and L. Cheng, *Stage pT1 bladder carcinoma: diagnostic criteria, pitfalls and prognostic significance.* Pathology, 2003. **35**(6): p. 484-91.
7. Smits, G., et al., *Microstaging of pT1 transitional cell carcinoma of the bladder: identification of subgroups with distinct risks of progression.* Urology, 1998. **52**(6): p. 1009-13; discussion 1013-4.
8. Cheng, L., et al., *Substaging of T1 bladder carcinoma based on the depth of invasion as measured by micrometer: A new proposal.* Cancer, 1999. **86**(6): p. 1035-43.
9. Pagano, F., et al., *Is stage pT4a (D1) reliable in assessing transitional cell carcinoma involvement of the prostate in patients with a concurrent bladder cancer? A necessary distinction for contiguous or noncontiguous involvement.* J Urol, 1996. **155**(1): p. 244-7.
10. Esrig, D., et al., *Transitional cell carcinoma involving the prostate with a proposed staging classification for stromal invasion.* J Urol, 1996. **156**(3): p. 1071-6.
11. Brausi, M., et al., *A review of current guidelines and best practice recommendations for the management of nonmuscle invasive bladder cancer by the International Bladder Cancer Group.* J Urol, 2011. **186**(6): p. 2158-67.
12. Fedewa, S.A., et al., *Incidence analyses of bladder cancer in the Nile delta region of Egypt.* Cancer Epidemiol, 2009. **33**(3-4): p. 176-81.
13. Moyer, V.A., *Screening for bladder cancer: U.S. Preventive Services Task Force recommendation statement.* Ann Intern Med, 2011. **155**(4): p. 246-51.
14. Keegan, K.A., M.J. Resnick, and P.E. Clark, *Multimodal therapies for muscle-invasive urothelial carcinoma of the bladder.* Curr Opin Oncol, 2012. **24**(3): p. 278-83.
15. Yafi, F.A., et al., *Contemporary outcomes of 2287 patients with bladder cancer who were treated with radical cystectomy: a Canadian multicentre experience.* BJU Int, 2011. **108**(4): p. 539-45.
16. Freedman, N.D., et al., *Association between smoking and risk of bladder cancer among men and women.* JAMA, 2011. **306**(7): p. 737-45.
17. Zeegers, M.P., et al., *The impact of characteristics of cigarette smoking on urinary tract cancer risk: a meta-analysis of epidemiologic studies.* Cancer, 2000. **89**(3): p. 630-9.
18. Baris, D., et al., *A case-control study of smoking and bladder cancer risk: emergent patterns over time.* J Natl Cancer Inst, 2009. **101**(22): p. 1553-61.
19. Griffiths, T.R., *Current perspectives in bladder cancer management.* Int J Clin Pract, 2012.
20. Khadra, M.H., et al., *A prospective analysis of 1,930 patients with hematuria to evaluate current diagnostic practice.* J Urol, 2000. **163**(2): p. 524-7.

21. Edwards, T.J., et al., *Patient-specific risk of undetected malignant disease after investigation for haematuria, based on a 4-year follow-up.* BJU Int, 2011. **107**(2): p. 247-52.
22. Mowatt, G., et al., *Systematic review of the clinical effectiveness and cost-effectiveness of photodynamic diagnosis and urine biomarkers (FISH, ImmunoCyt, NMP22) and cytology for the detection and follow-up of bladder cancer.* Health Technol Assess, 2010. **14**(4): p. 1-331, iii-iv.
23. Isfoss, B.L., *The sensitivity of fluorescent-light cystoscopy for the detection of carcinoma in situ (CIS) of the bladder: a meta-analysis with comments on gold standard.* BJU Int, 2011. **108**(11): p. 1703-7.
24. Raitanen, M.P., et al., *Routine follow-up cystoscopy in detection of recurrence in patients being monitored for bladder cancer.* Ann Chir Gynaecol, 2001. **90**(4): p. 261-5.
25. Hall, M.C., et al., *Guideline for the management of nonmuscle invasive bladder cancer (stages Ta, T1, and Tis): 2007 update.* J Urol, 2007. **178**(6): p. 2314-30.
26. Raghavan, D., et al., *Surgery and adjunctive chemotherapy for invasive bladder cancer.* Surg Oncol, 2002. **11**(1-2): p. 55-63.
27. Grossman, H.B., et al., *Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer.* N Engl J Med, 2003. **349**(9): p. 859-66.
28. Pruthi, R.S., et al., *A phase II trial of neoadjuvant erlotinib in patients with muscle-invasive bladder cancer undergoing radical cystectomy: clinical and pathological results.* BJU Int, 2010. **106**(3): p. 349-54.
29. Chester, J.D., et al., *Systemic chemotherapy for patients with bladder cancer--current controversies and future directions.* Cancer Treat Rev, 2004. **30**(4): p. 343-58.
30. Fahmy, T.M., et al., *Surface modification of biodegradable polyesters with fatty acid conjugates for improved drug targeting.* Biomaterials, 2005. **26**(28): p. 5727-36.
31. Thoren, P.E., et al., *The antennapedia peptide penetratin translocates across lipid bilayers - the first direct observation.* FEBS Lett, 2000. **482**(3): p. 265-8.
32. Tyagi, P., et al., *Recent advances in intravesical drug/gene delivery.* Mol Pharm, 2006. **3**(4): p. 369-79.
33. Herr, H.W. and A. Morales, *History of bacillus Calmette-Guerin and bladder cancer: an immunotherapy success story.* J Urol, 2008. **179**(1): p. 53-6.
34. Zlotta, A.R., et al., *What are the immunologically active components of bacille Calmette-Guerin in therapy of superficial bladder cancer?* Int J Cancer, 2000. **87**(6): p. 844-52.
35. Shelley, M.D., M.D. Mason, and H. Kynaston, *Intravesical therapy for superficial bladder cancer: a systematic review of randomised trials and meta-analyses.* Cancer Treat Rev, 2010. **36**(3): p. 195-205.
36. Sylvester, R.J., M.A. van der, and D.L. Lamm, *Intravesical bacillus Calmette-Guerin reduces the risk of progression in patients with superficial bladder cancer: a meta-analysis of the published results of randomized clinical trials.* J Urol, 2002. **168**(5): p. 1964-70.
37. Han, R.F. and J.G. Pan, *Can intravesical bacillus Calmette-Guerin reduce recurrence in patients with superficial bladder cancer? A meta-analysis of randomized trials.* Urology, 2006. **67**(6): p. 1216-23.
38. Chade, D.C., S.F. Shariat, and G. Dalbagni, *Intravesical therapy for urothelial carcinoma of the urinary bladder: a critical review.* Int Braz J Urol, 2009. **35**(6): p. 640-50; discussion 651.

39. Rischmann, P., et al., *BCG intravesical instillations: recommendations for side-effects management.* Eur Urol, 2000. **37 Suppl 1**: p. 33-6.
40. Gandhi, N.M., A. Morales, and D.L. Lamm, *Bacillus Calmette-Guerin immunotherapy for genitourinary cancer.* BJU Int, 2013.
41. Verweij, J. and H.M. Pinedo, *Mitomycin C: mechanism of action, usefulness and limitations.* Anticancer Drugs, 1990. **1**(1): p. 5-13.
42. Besarani, D. and M. Al-Akraa, *Immediate administration of intravesical mitomycin C after tumour resection for superficial bladder cancer.* BJU Int, 2006. **98**(1): p. 232-3.
43. Huland, H., et al., *Long-term mitomycin C instillation after transurethral resection of superficial bladder carcinoma: influence on recurrence, progression and survival.* J Urol, 1984. **132**(1): p. 27-9.
44. Huland, H. and U. Otto, *Mitomycin instillation to prevent recurrence of superficial bladder carcinoma. Results of a controlled, prospective study in 58 patients.* Eur Urol, 1983. **9**(2): p. 84-6.
45. Smaldone, M.C., et al., *Strategies to enhance the efficacy of intravescical therapy for non-muscle invasive bladder cancer.* Minerva Urol Nefrol, 2009. **61**(2): p. 71-89.
46. Shelley, M.D., et al., *Intravesical bacillus Calmette-Guerin is superior to mitomycin C in reducing tumour recurrence in high-risk superficial bladder cancer: a meta-analysis of randomized trials.* BJU Int, 2004. **93**(4): p. 485-90.
47. Bohle, A., D. Jocham, and P.R. Bock, *Intravesical bacillus Calmette-Guerin versus mitomycin C for superficial bladder cancer: a formal meta-analysis of comparative studies on recurrence and toxicity.* J Urol, 2003. **169**(1): p. 90-5.
48. Lamm, D.L., et al., *Apparent failure of current intravesical chemotherapy prophylaxis to influence the long-term course of superficial transitional cell carcinoma of the bladder.* J Urol, 1995. **153**(5): p. 1444-50.
49. Stadler, W.M., et al., *Phase II study of single-agent gemcitabine in previously untreated patients with metastatic urothelial cancer.* J Clin Oncol, 1997. **15**(11): p. 3394-8.
50. Ringel, I. and S.B. Horwitz, *Studies with RP 56976 (taxotere): a semisynthetic analogue of taxol.* J Natl Cancer Inst, 1991. **83**(4): p. 288-91.
51. McKiernan, J.M., et al., *Phase I trial of intravesical docetaxel in the management of superficial bladder cancer refractory to standard intravesical therapy.* J Clin Oncol, 2006. **24**(19): p. 3075-80.
52. Dokmanovic, M., C. Clarke, and P.A. Marks, *Histone deacetylase inhibitors: overview and perspectives.* Mol Cancer Res, 2007. **5**(10): p. 981-9.
53. Ellis, L. and R. Pili, *Histone Deacetylase Inhibitors: Advancing Therapeutic Strategies in Hematological and Solid Malignancies.* Pharmaceuticals (Basel), 2010. **3**(8): p. 2411-2469.
54. Marks, P.A. and R. Breslow, *Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug.* Nat Biotechnol, 2007. **25**(1): p. 84-90.
55. Giannantoni, A., et al., *New frontiers in intravesical therapies and drug delivery.* Eur Urol, 2006. **50**(6): p. 1183-93; discussion 1193.
56. GuhaSarkar, S. and R. Banerjee, *Intravesical drug delivery: Challenges, current status, opportunities and novel strategies.* J Control Release, 2010. **148**(2): p. 147-59.
57. Chuang, Y.C., et al., *Urodynamic and immunohistochemical evaluation of intravesical botulinum toxin A delivery using liposomes.* J Urol, 2009. **182**(2): p. 786-92.
58. Tyagi, P., et al., *Sustained intravesical drug delivery using thermosensitive hydrogel.* Pharm Res, 2004. **21**(5): p. 832-7.

59. Frangos, D.N., et al., *The development of liposomes containing interferon alpha for the intravesical therapy of human superficial bladder cancer.* J Urol, 1990. **143**(6): p. 1252-6.
60. Lazzeri, M., et al., *Intravesical infusion of resiniferatoxin by a temporary in situ drug delivery system to treat interstitial cystitis: a pilot study.* Eur Urol, 2004. **45**(1): p. 98-102.
61. Cheng, C.J. and W.M. Saltzman, *Nanomedicine: Downsizing tumour therapeutics.* Nat Nanotechnol, 2012. **7**(6): p. 346-7.
62. Di Stasi, S.M., et al., *Electromotive versus passive diffusion of mitomycin C into human bladder wall: concentration-depth profiles studies.* Cancer Res, 1999. **59**(19): p. 4912-8.
63. Di Stasi, S.M., et al., *Intravesical electromotive mitomycin C versus passive transport mitomycin C for high risk superficial bladder cancer: a prospective randomized study.* J Urol, 2003. **170**(3): p. 777-82.
64. Di Stasi, S.M., et al., *Intravesical electromotive drug administration of mitomycin-C for non-muscle invasive bladder cancer.* Arch Ital Urol Androl, 2008. **80**(4): p. 157-61.
65. Hildebrandt, B. and P. Wust, *Interactions between hyperthermia and cytotoxic drugs.* Cancer Treat Res, 2007. **134**: p. 185-93.
66. Hildebrandt, B. and P. Wust, *The biologic rationale of hyperthermia.* Cancer Treat Res, 2007. **134**: p. 171-84.
67. Wust, P., et al., *Hyperthermia in combined treatment of cancer.* Lancet Oncol, 2002. **3**(8): p. 487-97.
68. Fotopoulou, C., et al., *Regional abdominal hyperthermia combined with systemic chemotherapy for the treatment of patients with ovarian cancer relapse: Results of a pilot study.* Int J Hyperthermia, 2010. **26**(2): p. 118-26.
69. Tschoep-Lechner, K.E., et al., *Gemcitabine and cisplatin combined with regional hyperthermia as second-line treatment in patients with gemcitabine-refractory advanced pancreatic cancer.* Int J Hyperthermia, 2013. **29**(1): p. 8-16.
70. Triantopoulou, S., et al., *Radiotherapy in conjunction with superficial and intracavitary hyperthermia for the treatment of solid tumors: survival and thermal parameters.* Clin Transl Oncol, 2013. **15**(2): p. 95-105.
71. Glazer, E.S. and S.A. Curley, *The ongoing history of thermal therapy for cancer.* Surg Oncol Clin N Am, 2011. **20**(2): p. 229-35, vii.
72. Wust, P., et al., *Implications of clinical RF hyperthermia on protection limits in the RF range.* Health Phys, 2007. **92**(6): p. 565-73.
73. Coss, R.A., W.C. Dewey, and J.R. Bamburg, *Effects of hyperthermia on dividing Chinese hamster ovary cells and on microtubules in vitro.* Cancer Res, 1982. **42**(3): p. 1059-71.
74. Dewey, W.C., et al., *Heat-induced lethality and chromosomal damage in synchronized Chinese hamster cells treated with 5-bromodeoxyuridine.* Int J Radiat Biol Relat Stud Phys Chem Med, 1971. **20**(6): p. 505-20.
75. Westra, A. and W.C. Dewey, *Variation in sensitivity to heat shock during the cell-cycle of Chinese hamster cells in vitro.* Int J Radiat Biol Relat Stud Phys Chem Med, 1971. **19**(5): p. 467-77.
76. Iwata, K., et al., *Tumour pO2 can be increased markedly by mild hyperthermia.* Br J Cancer Suppl, 1996. **27**: p. S217-21.
77. Okajima, K., et al., *Tumor oxygenation after mild-temperature hyperthermia in combination with carbogen breathing: dependence on heat dose and tumor type.* Radiat Res, 1998. **149**(3): p. 294-9.
78. Vaupel, P.W., *The influence of tumor blood flow and microenvironmental factors on the efficacy of radiation, drugs and localized hyperthermia.* Klin Padiatr, 1997. **209**(4): p. 243-9.

79. Vaupel, P.W. and D.K. Kelleher, *Pathophysiological and vascular characteristics of tumours and their importance for hyperthermia: heterogeneity is the key issue.* Int J Hyperthermia, 2010. **26**(3): p. 211-23.
80. Appenheimer, M.M., et al., *Impact of fever-range thermal stress on lymphocyte-endothelial adhesion and lymphocyte trafficking.* Immunol Invest, 2005. **34**(3): p. 295-323.
81. Srivastava, P., *Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses.* Annu Rev Immunol, 2002. **20**: p. 395-425.
82. Chen, Q., et al., *Fever-range thermal stress promotes lymphocyte trafficking across high endothelial venules via an interleukin 6 trans-signaling mechanism.* Nat Immunol, 2006. **7**(12): p. 1299-308.
83. Chen, Q., et al., *Dynamic control of lymphocyte trafficking by fever-range thermal stress.* Cancer Immunol Immunother, 2006. **55**(3): p. 299-311.
84. Zanker, K.S. and J. Lange, *Whole body hyperthermia and natural killer cell activity.* Lancet, 1982. **1**(8280): p. 1079-80.
85. Dayanc, B.E., et al., *Dissecting the role of hyperthermia in natural killer cell mediated anti-tumor responses.* Int J Hyperthermia, 2008. **24**(1): p. 41-56.
86. Ostberg, J.R., et al., *Enhancement of natural killer (NK) cell cytotoxicity by fever-range thermal stress is dependent on NKG2D function and is associated with plasma membrane NKG2D clustering and increased expression of MICA on target cells.* J Leukoc Biol, 2007. **82**(5): p. 1322-31.
87. Lammers, R.J., et al., *The role of a combined regimen with intravesical chemotherapy and hyperthermia in the management of non-muscle-invasive bladder cancer: a systematic review.* Eur Urol, 2011. **60**(1): p. 81-93.
88. Matzkin, H., M.C. Rangel, and M.S. Soloway, *In vitro study of the effect of hyperthermia on normal bladder cell line and on five different transitional cell carcinoma cell lines.* J Urol, 1992. **147**(6): p. 1671-4.
89. Jen, C.P., C.T. Huang, and C.H. Tsai, *Supraphysiological thermal injury in different human bladder carcinoma cell lines.* Ann Biomed Eng, 2009. **37**(11): p. 2407-15.
90. van der Heijden, A.G., et al., *The effect of hyperthermia on mitomycin-C induced cytotoxicity in four human bladder cancer cell lines.* Eur Urol, 2004. **46**(5): p. 670-4.
91. van der Heijden, A.G., et al., *Effect of hyperthermia on the cytotoxicity of 4 chemotherapeutic agents currently used for the treatment of transitional cell carcinoma of the bladder: an in vitro study.* J Urol, 2005. **173**(4): p. 1375-80.
92. Rath-Wolfson, L., et al., *Combined intravesical hyperthermia and mitomycin chemotherapy: a preliminary in vivo study.* Int J Exp Pathol, 2003. **84**(3): p. 145-52.
93. Inman, B.A., et al., *The impact of temperature and urinary constituents on urine viscosity and its relevance to bladder hyperthermia treatment.* Int J Hyperthermia, 2013.
94. Colombo, R., *Combined treatment with local thermo-chemotherapy for non muscle invasive bladder cancer. The present role in the light of acquired data and preliminary cumulative clinical experiences.* Arch Ital Urol Androl, 2008. **80**(4): p. 149-56.
95. Colombo, R., et al., *Thermo-chemotherapy and electromotive drug administration of mitomycin C in superficial bladder cancer eradication. a pilot study on marker lesion.* Eur Urol, 2001. **39**(1): p. 95-100.
96. Colombo, R., et al., *Neoadjuvant combined microwave induced local hyperthermia and topical chemotherapy versus chemotherapy alone for superficial bladder cancer.* J Urol, 1996. **155**(4): p. 1227-32.

97. Colombo, R., et al., *Local microwave hyperthermia and intravesical chemotherapy as bladder sparing treatment for select multifocal and unresectable superficial bladder tumors.* J Urol, 1998. **159**(3): p. 783-7.
98. Colombo, R., et al., *Multicentric study comparing intravesical chemotherapy alone and with local microwave hyperthermia for prophylaxis of recurrence of superficial transitional cell carcinoma.* J Clin Oncol, 2003. **21**(23): p. 4270-6.
99. Colombo, R., et al., *A new approach using local combined microwave hyperthermia and chemotherapy in superficial transitional bladder carcinoma treatment.* J Urol, 1995. **153**(3 Pt 2): p. 959-63.
100. Colombo, R., et al., *Combination of intravesical chemotherapy and hyperthermia for the treatment of superficial bladder cancer: preliminary clinical experience.* Critical Reviews in Oncology/Hematology, 2003. **47**(2): p. 127-139.
101. Colombo, R., et al., *Long-term outcomes of a randomized controlled trial comparing thermochemotherapy with mitomycin-C alone as adjuvant treatment for non-muscle-invasive bladder cancer (NMIBC).* BJU Int, 2011. **107**(6): p. 912-8.
102. Gofrit, O.N., et al., *Combined local bladder hyperthermia and intravesical chemotherapy for the treatment of high-grade superficial bladder cancer.* Urology, 2004. **63**(3): p. 466-71.
103. Halachmi, S., et al., *Intravesical mitomycin C combined with hyperthermia for patients with T1G3 transitional cell carcinoma of the bladder.* Urol Oncol, 2011. **29**(3): p. 259-64.
104. Nativ, O., et al., *Combined thermo-chemotherapy for recurrent bladder cancer after bacillus Calmette-Guerin.* J Urol, 2009. **182**(4): p. 1313-7.
105. Paroni, R., et al., *Effect of local hyperthermia of the bladder on mitomycin C pharmacokinetics during intravesical chemotherapy for the treatment of superficial transitional cell carcinoma.* Br J Clin Pharmacol, 2001. **52**(3): p. 273-8.
106. Rigatti, P., A. Lev, and R. Colombo, *Combined intravesical chemotherapy with mitomycin C and local bladder microwave-induced hyperthermia as a preoperative therapy for superficial bladder tumors. A preliminary clinical study.* Eur Urol, 1991. **20**(3): p. 204-10.
107. van der Heijden, A.G., et al., *Preliminary European results of local microwave hyperthermia and chemotherapy treatment in intermediate or high risk superficial transitional cell carcinoma of the bladder.* Eur Urol, 2004. **46**(1): p. 65-71; discussion 71-2.
108. Alfred Witjes, J., et al., *Intravesical hyperthermia and mitomycin-C for carcinoma in situ of the urinary bladder: experience of the European Synergo working party.* World J Urol, 2009. **27**(3): p. 319-24.
109. Crook, T.J., et al., *A model of superficial bladder cancer using fluorescent tumour cells in an organ-culture system.* BJU Int, 2000. **86**(7): p. 886-93.
110. Crook, T.J., et al., *The effects of meglumine gamma linolenic acid (MeGLA) on an organ culture model of superficial bladder cancer.* Urol Res, 2002. **30**(1): p. 59-65.
111. Nitz, M.D., M.A. Harding, and D. Theodorescu, *Invasion and metastasis models for studying RhoGDI2 in bladder cancer.* Methods Enzymol, 2008. **439**: p. 219-33.
112. Sanchez-Carbayo, M., et al., *Molecular profiling of bladder cancer using cDNA microarrays: defining histogenesis and biological phenotypes.* Cancer Res, 2002. **62**(23): p. 6973-80.
113. Booth, C., et al., *Stromal and vascular invasion in an human in vitro bladder cancer model.* Lab Invest, 1997. **76**(6): p. 843-57.
114. O'Toole, C., et al., *Cellular immunity to human urinary bladder carcinoma. I. Correlation to clinical stage and radiotherapy.* Int J Cancer, 1972. **10**(1): p. 77-91.

115. Davies, G., W.G. Jiang, and M.D. Mason, *Cell-cell adhesion molecules and their associated proteins in bladder cancer cells and their role in mitogen induced cell-cell dissociation and invasion.* Anticancer Res, 1999. **19**(1A): p. 547-52.
116. Elliott, A.Y., et al., *Properties of cell lines established from transitional cell cancers of the human urinary tract.* Cancer Res, 1977. **37**(5): p. 1279-89.
117. Bubenik, J., et al., *Established cell line of urinary bladder carcinoma (T24) containing tumour-specific antigen.* Int J Cancer, 1973. **11**(3): p. 765-73.
118. Grossman, H.B., et al., *Improved growth of human urothelial carcinoma cell cultures.* J Urol, 1986. **136**(4): p. 953-9.
119. Nayak, S.K., C. O'Toole, and Z.H. Price, *A cell line from an anaplastic transitional cell carcinoma of human urinary bladder.* Br J Cancer, 1977. **35**(2): p. 142-51.
120. Mukerjee, A. and J.K. Vishwanatha, *Formulation, characterization and evaluation of curcumin-loaded PLGA nanospheres for cancer therapy.* Anticancer Res, 2009. **29**(10): p. 3867-75.
121. DeFoe, G.R., et al., *Lowest hematocrit on bypass and adverse outcomes associated with coronary artery bypass grafting. Northern New England Cardiovascular Disease Study Group.* Ann Thorac Surg, 2001. **71**(3): p. 769-76.