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Effects of hyperthermia in neutralising mechanisms of drug resistance in non-muscle-invasive bladder cancer

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\textbf{Abstract}
Non-muscle-invasive bladder cancer is a challenging disease, even given its superficial nature. It is prone to multiple recurrences and progression to muscle-invasive cancer. These features of this disease contribute significantly to reduced quality of life as well as creating significant morbidity and even mortality. Randomised trials demonstrate that when hyperthermia is added to conventional mitomycin-C treatment that local control rates and progression-free survival are substantially improved. In this review we consider how hyperthermia can exert such beneficial effects. Some of the mechanisms presented are theoretical, while others are facts. It is hoped that this review will contribute rationale for further examination of mechanisms, because an understanding of such mechanisms may lead to even better chemotherapeutic approaches, as well as potential biomarkers for predicting and monitoring treatment success.

\textbf{Introduction}
A series of previous studies in bladder cancer have shown that the non-muscle-invasive bladder cancer (NMIBC) recurrence rate is substantially reduced following radiofrequency-induced hyperthermia plus mitomycin-C (MMC), compared with ‘conventional’ intravesical MMC instillation [1]. Furthermore, the progression rate is low, even in patients presenting with carcinoma in situ (CIS), grade 3 (G3) tumours and patients failing BCG prior to radiofrequency-induced chemo-hyperthermia (RF-CH) treatment [2]. There are multiple potential reasons for the enhanced effects of this combination therapy. In this review we consider effects of hyperthermia on drug transport and on cell-killing efficiency. We believe that both play an important role in the success of this therapeutic combination.

\textbf{Drug transport barriers}
There are multiple barriers to effective delivery of drugs to cancers at cytotoxic concentrations. First, there are substantial barriers to deliver adequate concentrations of drug throughout the tumour volume. Second, cells have delivery barriers that must be circumvented. Drug has to be taken up by the cell in sufficient quantity to hit the intracellular target in order to be cytotoxic. Mechanisms of cellular drug resistance have been identified by many investigators and will be discussed.

\textbf{Barriers to transport of drugs from intravesical administration}
The challenges of drug delivery following intravenous administration have been discussed in excellent reviews [3,4]; but the basic concepts have not been discussed previously with reference to treatment of NMIBC, where drugs are typically administered intravesically. In this review we examine those features of drug transport from intravesical administration that are influenced by hyperthermia. We will focus mainly on discussion of MMC because it is a standard of care. However, the principles discussed are appropriate to any small molecule drug. In the discussion we will briefly examine issues related to intravenous drug administration, which is not currently used for NMIBC. Intravenous drug could be used if a method could be developed that could reduce systemic toxicity while maintaining cytotoxic drug levels in the bladder. We discuss thermosensitive liposomes as a platform through which this goal could theoretically be achieved.

\textbf{Sites of growth of NMIBC and rationale for standards of care}
NMIBC is located in the mucosa and can extend into the submucosa, but has not penetrated into the muscle layer. There is low probability for regional or distant metastasis with this presentation; therefore, it is considered a localised disease. Intravesical chemotherapy is used for NMIBC, since the...
The highest concentration of drug will be delivered directly to the site of tumour growth. In addition, one would not want to subject a patient who has local disease to the toxicities associated with systemically administered drug. However, there are substantial hurdles to achieving uniform cytotoxic drug delivery with NMIBC. Here we discuss the barriers and then how hyperthermia can improve drug delivery.

The standard of care for newly diagnosed NMIBC is transurethral resection of the tumour, followed by intravesical chemotherapy in low or intermediate risk, and immunotherapy with bacillus Calmette-Guérin (BCG) in intermediate- or high-risk NMIBC patients [5]. Although these therapies are initially effective, the 5-year recurrence rate is up to 80%, depending on the stage and grade of the tumour. The consequences of these recurrences are twofold. First, recurrences may require many resections. Eventually, this will lead to inability of the bladder to retain acceptable volumes of urine because the elasticity becomes compromised. This may require complete removal of the bladder, known as a cystectomy, because of functional problems. Cystectomy is associated with substantial morbidity. Even in experienced centres the 30 and 90 days perioperative mortality rate is respectively around 2 and 7% [6]. Second, approximately 15% of patients with high grade NMIBC will progress to muscle-invasive cancer, which also has a much worse prognosis compared to de novo muscle-invasive disease [7,8]. Thus, there is strong rationale to develop therapies that can lower recurrences and prevent progression to muscle-invasive disease.

One might assume that treatment efficacy would be maximised by using intravesical chemotherapy for NMIBC, since the drug will be in high concentration immediately adjacent to the mucosal surface. However, there are impediments to efficient drug delivery by intravesical means.

Glycosaminoglycans present a barrier to drug penetration

The inner lining of the bladder is covered by a thick layer of glycosaminoglycan (GAG), which is an effective barrier that limits passage of drugs to the tumour bladder cell surface [9,10]. In order to increase drug penetration across the GAG layer, one must either remove the GAG or enhance drug transport across it. A number of methods have been used to temporarily disrupt the GAG layer, as a means to enhance drug delivery. Examples include instillation of mild detergents and dimethylsulfoxide (DMSO) [9]. Additionally, drugs that inhibit GAG synthesis have been used to reduce the thickness of the layer [11]. Alternatively, glycosaminoglycan drug carriers have been shown to be taken up into GAG layers, increasing penetration of drug into tumour interstitium [12,13]. GAG has also been considered a barrier to effective therapy with the immunotherapeutic drug BCG [14]. Although these methods have resulted in improvements in antitumour effects of drugs in pre-clinical models, none have been approved for use in conjunction with intravesical chemotherapy.

Urothelium is the next layer of defence against drug penetration

The urothelium is composed of three types of cells: basal, intermediate and umbrella cells (Figure 1). The umbrella cells are very specialised to add another tight barrier to prevent urine and bacteria from passing from the bladder into adjacent tissues [15]. Tight protein-based junctions form between adjacent cells, which contribute to the impenetrability of this layer [16]. The lipid composition is comprised of cholesterol, phosphatidyl choline, phosphatidyl ethanolamine, and cerebroside. This composition makes the layer very water impermeable. To be able to deliver drugs to NMIBC, one must penetrate this impermeable layer.

Drug must penetrate through the mucosal surface to reach all tumour cells

MMC penetration across the bladder wall has been measured following intravesical chemotherapy in human patients [17]. Concentrations dropped from 120 μg/mL in the urine, to
Positive effects of hyperthermia on drug transport

Hyperthermia may exert beneficial effects related to the delivery limitations listed above. Some of these mechanisms will work directly on tumour cells as well as uroepithelial cells. It will become apparent that hyperthermia exerts beneficial effects to enhance drug transport across multiple barriers that emanate from intravesical therapy.

Effects on GAG and urothelial layers

Hyperthermia has been shown to transiently damage the urothelium, which can increase the rate of drug uptake into the bladder wall [18]. This results in increased drug absorption when radiofrequency-induced hyperthermia is applied [19]. This increase is even more pronounced in patients with unresected tumours.

Potential mechanisms for enhancement of drug transport across urothelium

Cytoskeleton

Hyperthermia has been reported to cause transient changes in the cytoskeleton of cells; this is largely attributed to the disassembly microtubules, microfilaments and actin fibres [20]. The cytoskeleton is known to be involved in maintenance of cell shape. If it collapses, this may weaken the tight junctions between the uroepithelial layers, thereby increasing permeability. We reported previously that hyperthermia increases microvascular permeability, opening up pores between endothelial cells of 100–400 microns in size [21]. Similar effects may occur with the urothelium. It is important to know that these cytoskeletal changes are transient and recover as cells induce the production of heat shock proteins in response to thermal stress [20].

Hyperthermia Enhances drug diffusion

The delivery of a small molecule through a tissue layer is dependent upon the diffusion coefficient. Diffusion coefficients are temperature dependent. According to the Stokes-Einstein equation, the diffusion coefficient increases by 14% when temperature increases from 37 to 43 °C, with this rise in temperature the hydraulic conductivity is also increased by 12% [22]. The increase in hydraulic conductivity is the result of enhanced permeability in the interstitium and reduced fluid viscosity. Hyperthermia (42 °C) has been shown to decrease urine viscosity by approximately 10%, as compared with 37 °C [23]. This combination of effects could contribute to increased drug penetration into the bladder wall.

Hyperthermia affects tissue structure

The effects of hyperthermia on cell density and shape may also contribute to enhanced drug transport. It has been reported that increased drug uptake into tumours can occur if efforts are made to reduce tumour cell density [3]. There are several reports indicating that hyperthermia can increase efficacy of a variety of chemotherapeutic agents. Apoptosis is a common mode of death following such treatments [24]. Ware et al. observed a decrease of 15% in cell volume 24 hours after radiofrequency (RF) treatment [25]. They concluded that decreases in individual cell area do play an important role in alteration of cell–cell junctions. There was also a loss of cell adhesion. Furthermore, radiofrequency alters the surface roughness of malignant cells. This change will influence the interactions between chemotherapy and the cell membrane. Further, hyperthermia increased tunnelling nanotube formation, which results in more cell-to-cell interactions. Tunnelling nanotube formation has been shown to enhance particle trafficking in
cancer cells. These observations by Ware were performed in circumstances where temperature was controlled to <39°C. It is unclear whether similar effects would be observed if therapeutic temperatures were induced with RF. Induction of a wave of apoptosis after thermochemotherapy could reduce tumour cell packing density, thereby enabling more effective administration of a second drug [3]. To our knowledge, this therapeutic strategy has not been attempted for treatment of NMIBC. Similarly, use of agents that can modify the density of the extracellular matrix can also improve drug penetration [3]. To our knowledge, however, there have not been any reports indicating whether hyperthermia changes extracellular matrix to improve drug transport.

**Potential negative effects of hyperthermia on drug transport**

**Tissue oedema**

Transient oedema was observed in sheep bladder tissues after hyperthermia [18]. The diffusion distance of a small molecule is proportional to L²/D, where L is the distance and D is the diffusion coefficient. Bladder oedema could increase the thickness of the bladder wall, which could reduce drug delivery by increasing the distance over which drug must diffuse to reach target tumour cells. Oedema would also reduce the local drug concentration, which could reduce the drug concentration gradient. These two effects could reduce drug transport across the bladder wall. On the other hand, the diffusion of drug can increase because tissue density is decreased in the presence of oedema. This would tend to increase the diffusion distance. It is difficult to predict the net effect of hyperthermia on drug diffusion, given these offsetting forces.

**Urine production during heating dilutes drug concentration in bladder**

Dilution of drug by urine can reduce the exposure of the bladder wall to drug. In a human clinical trial, urine concentrations of MMC were measured during and after treatment. Patients were treated with and without intravesical hyperthermia with a microwave antenna. Patients who received hyperthermia produced more urine, thereby resulting in more dilution of drug than the control patients who were not heated. For example, at an intravesical dose of 40 mg the urine concentration dropped by fourfold in the heated group, compared with 2.2-fold in the control group [19].

**Increased perfusion may remove drug from target volume**

Even though NMIBC is superficial, these tumours can be vascularised [26]. There is additional vasculature in the muscle layer, deep in relation to the position of the tumour. Hyperthermia at 43°C for up to 2 h increases skeletal muscle perfusion of rats by 3.5- to sixfold [27]. Similar effects were observed in canine thigh muscle in response to thermal stress [28]. The tissue response to thermal stress follows a biphasic curve, where perfusion increases by approximately twofold between 37 and 42°C. Above 42°C, perfusion increases by approximately a factor of 3 for every 1°C rise in temperature [28]. Multiple mechanisms have been postulated to be involved in the regulation of perfusion upon thermal stress. These include enhanced release of vasoactive substances, such as bradykinin and prostacyclin. In addition, enhanced NO production by vascular endothelium could contribute [28]. The muscularis of the bladder is composed of smooth muscle, so one has to consider whether there is precedent for hyperthermia having a direct effect on perfusion of this organ. Activation of TRP (transient receptor potential) channels may be involved in regulating smooth muscle vasodilation upon heat stress [29,30]. Perfusion in tumours can increase as well by as much as 2-fold [27]. The increase in perfusion in both tumour and muscle of the bladder wall could enhance drug removal from the tumour, where it could be redistributed systemically. Paroni et al. reported that systemic levels of MMC were increased following intravesical administration in patients who received local heating of the bladder [19]. On the other hand, this observation is consistent with the concept that systemic removal of drug is increased with local hyperthermia delivered to the bladder. On the other hand, the fact that there is an increase in systemic levels of drug means that the drug was penetrating deep enough to be picked up by the blood vessels. Presumably, this is indirect evidence for greater tumour cell exposure to drug as well, since the drug would have to pass through multiple cell layers to reach the deeper vasculature. The systemic levels of drug achieved with hyperthermia were below those that are known to cause normal tissue toxicity.

Theoretically, exposure of tumour cells to drug could be increased if hyperthermia causes vascular damage in tumours. Such damage would reduce systemic uptake of drug. In early studies there was the suggestion that the vasculature of tumours is more thermally sensitive than that of normal tissue, with thresholds of thermal damage in the range of 42–43°C for 1 h exposure [27]. Most of those data were based on observations in rodents. More recent studies have indicated that the vasculature of human/canine tumours is more resilient and that vascular shut down does not occur below temperatures of 44°C for 1 h. For example, in companion canine soft tissue sarcomas, intratumoural temperatures in the range of 40–43°C resulted in increased perfusion and oxygenation at 24 h post-hyperthermia treatment [31]. In contrast, temperatures >44°C resulted in reduced perfusion and oxygenation, most likely as a result of vascular damage. Similar results were observed in women with locally advanced breast cancer who were treated with a combination of hyperthermia, radiotherapy and chemotherapy. Further, reoxygenation was associated with better clinical response in the locally advanced breast cancer study [32]. The prescribed temperature range for bladder heating is in the range of 42–43°C [33,34]. Thus it is unlikely that vascular damage to the tumour or normal tissue vasculature is a consequence. If vascular damage did occur, the consequence could easily result in less drug uptake by the tumour. This could occur because such damage would not maintain the concentration...
gradient of drug across the tumour – should this occur, Fickian diffusion could be reduced.

**Hyperthermia increases drug uptake by, and cytotoxicity of, tumour cells**

Hyperthermia has been reported to increase cellular uptake of some drugs; this increase has been considered one of the factors that leads to enhanced antitumour effects. Cisplatin, MMC, gemcitabine, doxorubicin and epirubicin, are all used in intravesicle treatment of bladder cancer. We will discuss the effects of hyperthermia on cellular transport of these drugs in more detail.

The permeability of lipid bilayers is affected by the order of acyl chains of phospholipids. Increasing temperature reduces the order of the acyl chains, thereby increasing permeability. Increases in membrane fluidity have been associated with increases in drug uptake by cells, including doxorubicin [35], and cisplatin [36]. However, the effects of hyperthermia on cell membrane fluidity are not the entire explanation for enhanced uptake for all drugs.

Hyperthermia inhibits DNA damage repair. The most important lethal target is the double strand break, which is repaired by two mechanisms: non-homologous end joining and homologous recombination. Both of these mechanisms of repair are inhibited by hyperthermia [37]. Important thermal targets include DNA polymerases and poly(ADP-ribose)-polymerase-1 (PARP) [38,39]. PARP functions to direct DNA repair enzymes to the location of strand breaks. Thermal inhibition of BRCA2, an enzyme responsible for initiation of DNA repair, combined with inhibition of PARP is synthetically lethal to target cells. Hyperthermia has also been reported to inhibit recombination repair of stalled DNA replication forks, which are the primary target lesion of gemcitabine [40].

**Cisplatin**

Alberts was one of the first investigators to report that hyperthermia (42°C) increased cytotoxicity of cisplatin, in vivo, using a mouse leukaemia line [41]. Using radiolabelled cisplatin, he demonstrated that hyperthermia increased cellular uptake of cisplatin. Meyn et al. reported that hyperthermia (43°C) of CHO cells increased the extent of lethal DNA crosslinks created by cisplatin [42]. The dose modifying factor, which is the ratio of drug doses to achieve an equal level of cytotoxicity at 37°C versus 43°C, was nearly a factor of 6. They hypothesised that the increase in crosslinks could be a result of inhibition of DNA damage repair, but the rate of disappearance of crosslinks was the same for both temperatures. They concluded that the increase in cytotoxicity of cisplatin by hyperthermia was most likely caused by an increase in drug uptake. Los et al., demonstrated that regional hyperthermia (41°C) increased drug uptake by over fourfold, increased DNA crosslinking by 2.5-fold and increased in vivo efficacy of cisplatin, using a rat colon carcinoma line [43]. Los also reported that the threshold for enhancement of cisplatin uptake by hyperthermia was 38.5°C [44]. Using isolated DNA, they were able to show that the rate of cisplatin binding to DNA was not temperature dependent. Thus, it is reasonable to conclude that the enhancement in cytotoxicity caused by hyperthermia is a result of increased cellular uptake, leading to an increase in DNA crosslinks. It has been recently reported that the mechanism of enhanced cisplatin uptake is related to the function of the copper transporter, CTR1. Hyperthermia increases the extent of CTR1 multimerisation, which creates a membrane pore to facilitate cisplatin uptake [45]. Wallner and Li demonstrated that the greatest effectiveness of cisplatin with hyperthermia occurs when the two are given simultaneously, with a hyperthermia treatment period of 1 h [46]. Longer heating times were less effective. This observation fits well with the expected use of cisplatin with hyperthermia, since the drug would be instilled into the bladder and hyperthermia could commence quickly after instillation.

**Mitomycin C**

There are several references related to the combination of hyperthermia with MMC, dating back to the early 1980s [47–52]. Wallner et al. examined the effects of hyperthermia on cytotoxicity of MMC in drug-sensitive and drug-resistant Chinese hamster ovary (CHO) cell lines [53]. Dose-modifying factors for 42°C and 43°C heating with MMC ranged from 1.3–2.0 and 2.6–3.8, respectively. The magnitude of the dose-modifying factor was independent of whether the cells were drug sensitive or drug resistant. Hyperthermia (43°C) increased cellular uptake of MMC by 78% versus 27% for drug-sensitive versus drug-resistant cell lines. Like cisplatin, simultaneous administration of drug and hyperthermia was the most efficacious.

Teicher et al. examined the effects of hyperthermia (41, 42 and 43°C) on cytotoxicity of MMC, under aerobic and hypoxic conditions [49]. Although they did not measure drug uptake, they found that the rate of formation of active drug increased remarkably when hyperthermia was added to drug. Further, they demonstrated that the thermal enhancement of MMC killing was greater in hypoxic cells than in aerobic cells. Considering that bladder cancer can be hypoxic, this provides very strong rationale for using MMC in combination with hyperthermia [54].

**Gemcitabine**

We were able to identify one paper in which intratumoural gemcitabine concentrations were measured after hyperthermia treatment (42°C). These authors did not observe any increase in gemcitabine uptake with hyperthermia [55]. We were unable to identify any in vitro studies that examined the question of whether hyperthermia augmented drug uptake.

Hyperthermia (43°C) has been shown to enhance the cytotoxicity of gemcitabine, however. In one study, two pancreatic cell lines were assessed for cell viability by the WST-8 assay, which reports on cell mass after treatment [56]. This assay reflects the balance between cell death and cell proliferation. Reduction in WST-8 signal occurred in two scenarios: when heat was administered 24 h prior to drug, or when the two treatments were given simultaneously. The enhancement
in cell killing was associated with thermal inhibition of NFκB activation by tumour cells.

Vertrees et al. examined the combination of gemcitabine and hyperthermia (temperatures between 40 and 44 °C for periods ranging from 60 to 180 min) using a human non-small-cell lung cancer line [57]. The optimal sequence was as discussed above, where hyperthermia was administered 24 h prior to drug treatment. The optimal temperature was found to be 43.5 °C. Using isobologram analysis of in vitro survival data, they demonstrated that this combination led to synergistic killing. The mechanism of killing appeared to be related to activation of apoptosis. Interestingly, however, they were able to demonstrate a significant prolongation of tumour regrowth when tumour-bearing animals were exposed to 40 °C for 1 h, 24 h prior to gemcitabine treatment. Haveman also reported that a 24-h delay between hyperthermia treatment and gemcitabine yielded the greatest level of cell killing as assessed by clonogenic assay [58].

Mohamed et al. reported that administration of gemcitabine immediately prior to heating at 41.5 °C led to enhanced antitumour effect [59]. Close examination of these data, however, shows that the enhancement was quite small in magnitude.

In contrast to most of the results reported above suggesting that a 24-h period between heating and administration of gemcitabine was required for optimal enhanced cytotoxicity, Raoof et al. recently reported that enhanced antitumour effect in a hepatoma tumour line was seen regardless of whether gemcitabine was given 22 h after or immediately after heating [40].

Given the recent results of Raoof et al., it would seem prudent to conduct additional studies, using thermal conditions seen with bladder heating, to determine whether heating immediately prior to drug administration would yield positive effects. Logistically, this would much easier to translate to the clinic, than requiring a 24-h period between the two treatments.

**Doxorubicin**

There are several reports in the literature demonstrating that hyperthermia increases doxorubicin uptake and retention by cells. In some reports, hyperthermia enhanced doxorubicin cytotoxicity. Further, hyperthermia facilitated uptake into both drug-sensitive and drug-resistant tumour cells. Key conceptual papers are discussed. Unlike cisplatin and MMC, there is some controversy as to what the ideal temperature would be to facilitate enhanced drug uptake and cell killing.

Hahn was the first investigator to report that hyperthermia increases doxorubicin uptake by tumour cells [60]. Using the CHO cell line, they observed little enhancement of doxorubicin cytotoxicity at 41 °C, but substantial enhancement at 42 °C. Using an in vivo, in vitro clonogenic assay of the EMT6 tumour grown in mice, they demonstrated that 43 °C heating yielded a dose-modifying factor for cell survival of nearly six. Concomitantly, they observed a threefold increase in fluorescence intensity of doxorubicin in 43 °C heated cells, compared with 37 °C controls.

Bates and McKillop examined the effects of hyperthermia (39–45 °C) on doxorubicin cytotoxicity of drug-sensitive and drug-resistant CHO cells [61]. Hyperthermia (43 °C) enhanced the rate of uptake of radiolabelled doxorubicin by approximately 80%. Enhancement of doxorubicin cytotoxicity of the drug sensitive line was seen at temperatures >39 °C (two- to threefold increase in cell killing compared with 37 °C), but the greatest enhancements in cytotoxicity were seen at temperatures of ≥42 °C (relative increase in cell killing by factors of 10, 50 and 100 at T = 42, 43 and 45 °C, respectively). In contrast, hyperthermia administration, even up to temperatures of 47 °C, did not increase drug cytotoxicity of the drug-resistant line.

Using an elegant confocal approach, Kawai et al., demonstrated that 43 °C hyperthermia increased the accumulation of doxorubicin into the nuclei of both drug-sensitive and drug-resistant oesophageal carcinoma cell lines [62]. The enhancement in uptake was 4.3 and 7.2-fold for the drug-sensitive and drug-resistant lines. Hyperthermia enhanced the cytotoxicity of doxorubicin in both the wild-type and drug-resistant cell lines. In fact, the enhancement of cytotoxicity was equivalent between the two cell types in this study.

**Epirubicin**

We were unable to identify any papers that examined the effects of hyperthermia on epirubicin drug uptake. The toxicity of epirubicin-treated cells under hyperthermic conditions was examined in a paper that is described more fully below.

**Hyperthermia inactivates membrane drug pumps and intracellular detoxification mechanisms**

Inactivation of detoxification mechanisms could increase drug uptake by urothelium for transport to deeper tumour cell layers. These same mechanisms will be important for enhancing tumour cell killing as well. Such mechanisms include ATP-binding cassette (ABC) drug pumps, such as p-glycoprotein [63], and antioxidant mechanisms such as glutathione [64,65].

Hyperthermia has been reported to inactivate multiple drug detoxification mechanisms. From a phenomenological point of view, Wallner et al. were among the first to report that hyperthermia could at least partially reverse drug resistance to MMC. They demonstrated that drug uptake was increased by heat in wild-type and MMC-resistant CHO cells [53]. The increases in drug uptake were associated with enhanced cytotoxicity. It has also been reported that hyperthermia reduces cell membrane localisation of multidrug resistance proteins, when combined with MMC [66]. If cellular uptake of drug is increased as a result of inactivating pumps, this will contribute to maintaining a drug concentration gradient in the tissue. Small molecules such as MMC diffuse through tissue, driven by their concentration gradient [67]. Once the drug enters a cell, it no longer contributes to the gradient.
Glutathione is the primary intracellular modifier of oxidative stress. It has been shown to play a role in reducing cytotoxicity of a variety of drugs, including MMC [68]. Laskowitz et al. demonstrated that hyperthermia reduced intracellular glutathione levels and that this was associated with enhanced in vivo activity of melphalan against a drug-resistant rhabdomyosarcoma xenograft [69]. The reduction in glutathione levels may in part be due to an increase in oxidative stress that has been associated with hyperthermia treatment [70,71].

**Rationale for intravenous drug administration in the treatment of NMIBC**

Intravenous drug administration has not been considered clinically viable for NMIBC, because of the low probability that patients that present with NMIBC would have spread of their disease beyond the confines of the bladder wall. Under the mantra of 'doing no harm', it is difficult to justify administering a toxic drug to such a population of patients, since such drugs have substantial systemic toxicities. Furthermore it is also important to realise that the drug dose administered in the bladder is much higher than the dose that can be used intravenously.

Theoretically, however, administration of systemic drug has some merit, particularly considering the fact that NMIBC often has a vascular supply. If one were able to administer a drug systemically, but stimulate its action selectively in the bladder, then systemic toxicities could be minimised. One such approach might be to use a thermally sensitive drug carrier, such as a liposome or other nanoparticle [72,73]. In this case,
hyperthermia could be used to stimulate enhanced drug release in the bladder. Clinical trials with a doxorubicin-containing thermosensitive liposome indicate that the systemic toxicities are no higher or different than those of free drug, when given at the maximally tolerated dose [74,75]. However, local hyperthermia triggers intravascular drug release in the heated zone. This mechanism enhances drug delivery to the tumour many fold, increases drug diffusion distance from the vasculature and enhances antitumour activity of several drugs, including doxorubicin, cisplatin and gemcitabine [76–80]. Because of the differential in systemic versus local effects of these formulations, one could administer less than the maximally tolerated dose and still enhance drug delivery to the bladder tumour. However, this approach still has to be considered experimental.

Summary regarding temperature dependence of drug uptake and cytotoxicity

Since the target temperature for intravesical hyperthermia is 41–42 °C, we will summarise the salient points about how temperatures in this range can alter drug uptake and cytotoxicity. For both cisplatin and MMC, heating in this temperature range will increase drug uptake and enhance cell killing by substantial amounts. The most efficient cell killing occurs if the drugs and heat are given simultaneously.
The effects of hyperthermia on doxorubicin accumulation and cytotoxicity are somewhat more complicated. The literature shows that minor enhancements in cell killing can be seen at 41°C, but the enhancement is much greater at >42°C. Therefore, when using doxorubicin it may be advised to push intravesical temperatures to 42°C or even slightly above.

Currently, one report indicates that hyperthermia does not increase gemcitabine uptake; but there are several reports showing that gemcitabine cytotoxicity is enhanced by hyperthermia. In most studies, the greatest enhancement occurs if the drug is given 24h after heating. However, recent data suggest that similar antitumour effects can be seen when gemcitabine is given immediately after hyperthermia.

**Direct comparison of hyperthermia combined with four drugs**

In 2005 we reported the cytotoxicity results of MMC, epirubicin, gemcitabine and EO9 with or without hyperthermia in four human bladder cancer cell lines (RT4, RT112, 253J and T24) [81]. MMC is an antibiotic antitumour agent (molecular weight (MW) 334 g/mol) that interacts with DNA. Cytotoxicity is a direct result of DNA damage. MMC is the most frequently used intravesical chemotherapeutic agent. Epirubicin is also a frequently used agent in NMIBC. It is an analogue of the antibiotic antitumour agent doxorubicin (MW 544 g/mol). It binds to DNA and inhibits nucleic acid synthesis and function. An intravesically less frequently used drug is gemcitabine, a deoxycytidine analogue (MW 300 g/mol). After intracellular activation, the active metabolite is incorporated in the DNA and results in DNA synthesis inhibition. The fourth agent studied, EO9, is a bio-reductive alkylating indoloquinone (MW 288 g/mol). The activity of EO9 is presumably related to the presence of DT-diaphorase enzyme in tumour tissue. This bioreductive drug showed good activity in vitro but failed when given intravenously. The reason for this poor efficacy in vivo is the rapid clearance from the blood stream. However, this is of no relevance when used intravesically [82].

In our preclinical study, cells were treated for 60 min with increasing concentrations of the four chemotherapeutic agents mentioned at 37 or 43°C. Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. An inverse relation between cell proliferation and drug concentrations was demonstrated. EO9 proved to be the most potent agent at both temperatures (Figure 3). In this experiment hyperthermia alone did not decrease cell proliferation. However, a synergistic effect of hyperthermia on decreased cell proliferation was demonstrated in all cell lines and chemotherapeutic agents used, although each reached a maximum at a different drug concentration (Figure 4). The extent of inhibition was different between the drugs. The highest level of synergism was seen in cells treated with epirubicin, and this occurred at lower drug concentrations. One of the explanations for increased effect was a higher uptake of the drugs. A counterargument is the limited effect on cell death shown in gemcitabine, a drug with a much lower molecular weight than epirubicin.

A more plausible explanation for the increased effect is a drug-specific activation under hyperthermic conditions.

**Summary and conclusions**

The remarkable success of intravesical MMC with hyperthermia in the treatment of NMIBC lends strong rationale for moving toward making this therapy a standard of care. However, the mechanisms underlying the success of this combination therapy are only partially understood. We have provided some clues as to what may be in play. More work needs to be done to investigate mechanisms that lead to enhanced antitumour effect when combining hyperthermia with agents that can be used for intravesical treatment.

As a final point, there have been several clinical reports using MMC and hyperthermia to treat patients whose tumours have recurred after BCG therapy [2]. It is curious, however, that we were unable to identify any reports testing whether hyperthermia augments the efficacy of BCG. Since the mechanism underlying efficacy of BCG is thought to be immunological, one might believe hyperthermia with BCG would prove beneficial. There is strong evidence that heat shock proteins play an important role in the efficacy of BCG [83]. Hyperthermia increases the expression of heat shock proteins and enhances antigen presentation by dendritic cells [84,85]. Via a range of effects, hyperthermia boosts the immune system to increase antitumour efficacy [86–91]. These effects may extend to tumour-bearing areas that are not effectively heated, via abscopal immune mediated effects [92].

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