

## Optimizing a Novel Regional Chemotherapeutic Agent against Melanoma: Hyperthermia-Induced Enhancement of Temozolomide Cytotoxicity

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**Abstract Purpose:** Previous preclinical studies have shown that regional temozolomide therapy via isolated limb infusion is more effective than melphalan, the current drug of choice for regional chemotherapy for advanced extremity melanoma. The aim of this study was to determine whether hyperthermia could further augment the efficacy of temozolomide, an alkylating agent, against melanoma and improve its therapeutic index in a rat model of isolated limb infusion.

**Experimental Design:** Athymic rats bearing s.c. human melanoma xenografts (DM6) in their hind limbs were randomized to a 15-minute isolated limb infusion procedure with or without temozolomide at room temperature, normothermic (37.5°C), or hyperthermic (43°C) conditions.

**Results:** The concomitant administration of hyperthermia during an infusion with temozolomide led to the greatest increase in tumor growth delay, decreased proliferative index, and increased cell death. Isolated limb infusion treatment with a low dose (350 mg/kg) of temozolomide was ineffective at producing tumor growth delay ( $P = 0.07$ ). Similarly, temozolomide infusion under normothermia yielded minimal tumor growth delay ( $P = 0.08$ ). In contrast, the combination of hyperthermia plus temozolomide treatment produced marked tumor growth delay of 10.4 days ( $P = 0.02$ ) with minimal toxicity. The addition of heat to temozolomide treatment yielded the smallest proliferative index ( $P = 0.001$ ), while markedly increasing the level of apoptosis 48 hours after isolated limb infusion.

**Conclusion:** This study, the first to examine the interaction between hyperthermia and temozolomide, shows a strong, synergistic antitumor effect when hyperthermia is combined with temozolomide for regional treatment of melanoma confined to an extremity. The mechanism of this synergy seems to be through an augmentation, by hyperthermia, of the antiproliferative and proapoptotic effects of temozolomide.

Isolated limb perfusion and isolated limb infusion have emerged as effective forms of treatment for regionally advanced melanoma confined to an extremity. These procedures address all in-transit lesions confined to an extremity in a single procedure by supplying a regional concentration of chemotherapeutic agents at least 10 times higher than that reached after systemic administration, without the systemic side effects.

Hyperthermia is widely used in conjunction with isolated limb perfusion and infusion to improve the response to the regional chemotherapy. This is usually done by heating the

perfusate (or infusate) to 39°C to 41°C and by applying external warming blankets to prevent heat loss. Hyperthermic isolated limb perfusion was introduced based on *in vitro* evidence of a synergistic, cytotoxic interaction between heat and melphalan, the alkylating agent that is the current drug of choice for regional therapy of melanoma (1). Melphalan has been extensively studied for its efficacy in hyperthermic isolated limb perfusion, with response rates in the 80% range with 40% to 80% being complete responders (2, 3).

However, a recent, large multicenter phase III randomized trial comparing melphalan to melphalan plus tumor necrosis factor found complete response rates to be only 30% with half of the complete responders recurring at 6 months (4). Although a significant subset of patients achieve a durable complete response attesting to the potential efficacy of the regional treatment strategy, over half the patients are left with residual disease after treatment and ~30% to 50% of complete responders recur relatively quickly. Therefore, significant opportunities exist for optimizing this type of treatment. New regional treatment strategies have included looking at different chemotherapy agents like temozolomide (5), modulating drug resistance proteins to improve treatment response (6, 7),

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developing ways drugs can be given repetitively (8), and optimizing how drugs are given such as in conjunction with varying degrees of hyperthermia (9).

Our recent work with regional infusion therapy using a rodent animal model of in-transit melanoma showed that temozolomide is an effective alternative to melphalan for regional chemotherapy (5). Temozolomide is an alkylating chemotherapeutic agent with a broad-spectrum antitumor activity and relatively low toxicity (10). Temozolomide is similar to dacarbazine, the standard chemotherapy drug used systemically in metastatic melanoma, in that both temozolomide and dacarbazine convert to the same active metabolite 5-(3-methyltriazene-1-yl)imidazole-4-carboximide (10). However, dacarbazine requires hepatic metabolism for activation whereas temozolomide spontaneously degrades to 5-(3-methyltriazene-1-yl)imidazole-4-carboximide under physiologic conditions (10). Interestingly, we have shown, using a preclinical animal model, that temozolomide can be used intra-arterially and that the antitumor activity of temozolomide was more effective when applied regionally (as opposed to systemically), with better response rates than melphalan (5).

Although many alkylating agents, including melphalan, are known to show a synergistic antitumor activity when combined with hyperthermia, temozolomide has not been studied to see whether or not hyperthermia could augment its therapeutic effects. Our specific aims for this study were (a) to examine whether the therapeutic index of temozolomide can be improved through the administration of regional hyperthermia using an *in vivo* animal model and (b) to elucidate the mechanisms responsible for any temozolomide-hyperthermia interaction detected in this model.

## Materials and Methods

**Cell culture and drugs.** Human melanoma cell line DM6 was provided by Dr. Hilliard Seigler (Duke University Medical Center, Durham, NC). DM6 cells were maintained in tissue culture with Iscove's modified Dulbecco's medium supplemented with 5% fetal bovine serum, L-glutamine, penicillin, and streptomycin.

Temozolomide was provided by Dr. W. Robert Bishop (Schering-Plow Research Institute, Kenilworth, NJ). A stock solution of 4 mg/mL temozolomide in 10% DMSO in PBS was prepared immediately before surgery. The infusate was prepared by further dilution of temozolomide stock solution according to the given dosing regimen.

**Animals and tumor cell implantation.** Female athymic nude rats (age 5-7 weeks; RHU-M, Harlan, Indianapolis, IN) were housed under standard conditions. The animals were first administered 500 cGy whole body radiation while anesthetized with 0.1 mL ketamine (Ketaset III, Fort Dodge Animal Health, Fort Dodge, IA) administered *i.p.* Within 2 days after irradiation, the rats were injected *s.c.* in the lateral portion of the right distal hind limb with  $5 \times 10^6$  DM6 cells. A total of 69 rats were used in this study. All animal experimental protocols were approved by the Duke University Medical Center Institutional Animal Care and Use Committee.

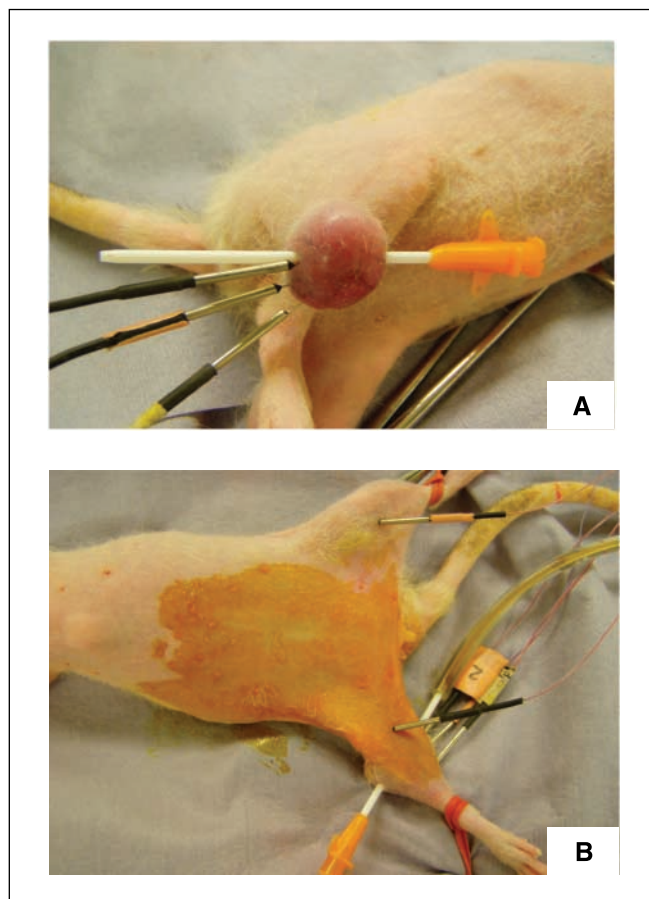
**Xenograft therapy.** Once the tumors reached 12 to 15 mm in maximal dimension (~3 weeks following tumor inoculation), rats were randomly assigned to one of the treatment subgroups, with six rats used to evaluate each dosage or specific condition. Rats were treated with temozolomide administered via isolated limb infusion at doses of 350 or 1,500 mg/kg of infused limb weight at room temperature or under controlled normothermia or hyperthermia. The limb weight was estimated to be 10% of the body weight of rat (11). The two temozolomide dosages used in this study were based on our previous

work, which showed the therapeutic dosage ranges of temozolomide in the rodent isolated limb infusion model (5). Isolated limb infusion was also done using 10% DMSO (in PBS) as the infusate with or without heat to serve as controls.

**Isolated limb infusion technique.** Rats were anesthetized with 0.05 mL of ketamine administered *s.c.* before undergoing isolated limb infusion. The animals were also maintained on 1.0% inhaled isoflurane anesthesia during the procedure.

Through an inguinal incision, the right femoral artery and vein were isolated using an operating microscope for the dissection. A tourniquet was placed loosely around the proximal thigh and was threaded underneath the inguinal ligament. After ligating the proximal femoral artery and vein, each vessel was cannulated with 25 G catheters. The arterial catheter was attached to a peristaltic pump (Master Flex model 7524-00, Cole-Parmer Instrument Co., Vernon Hills, IL). The venous cannula drained into a reservoir. The thigh tourniquet was then tightened and isolated limb infusion was done for 15 minutes with a flow rate of 1.5 mL/min, followed by a 1-minute washout infusion with 0.9% sodium chloride. The pump was then stopped, the tourniquet was loosened, and the femoral cannulas were removed. The vessels were ligated distally and the skin incision was approximated.

**Application of heat.** Hyperthermia was induced as described by Abdel-Wahab et al. (9). In brief, a 14 G catheter was placed through the tumor center immediately before surgery as shown in Fig. 1. Heat was initiated by passing heated water through the catheter at the onset of surgery (the time of the incision) and was discontinued at the conclusion of surgery (the conclusion of the infusion). A temperature



**Fig. 1.** Heat was initiated by passing heated water through the catheter inserted in the tumor center (A). Temperature was measured with a k-type hypodermic thermocouple inserted ~1, 3, and 7 mm from the tumor center as well as in the muscles of both affected and opposite unaffected leg (A and B).

of 43°C was used as the heating surface of the catheter in the hyperthermic isolated limb infusion group so that the majority of the xenograft would be heated to a range between 38°C and 43°C, which is comparable with temperature applied clinically in hyperthermic isolated limb perfusion or infusion (12, 13). For normothermic isolated limb infusion, a temperature of 37.5°C was used for the heating surface of the catheter to ensure that the tumor temperature would remain above 35°C without being heated to hyperthermic condition. Temperature profiles during the procedure were measured with a k-type hypodermic thermocouple (Omega Probes, Stamford, CT) inserted ~1, 3, and 7 mm away from the tumor center as well as in the muscles of both the affected and the opposite unaffected leg (Fig. 1). The conventional hyperthermic dosage, CEM43°C (cumulative number of equivalent minutes at 43°C), for each survival surgery was calculated, as described previously (14) according to the following equation:

$$\text{CEM43}^\circ\text{C} = \text{tR}^{(43-T)}$$

Average total CEM43°C was compared between all treatment groups where heat was applied using Wilcoxon rank sum test to ensure that equivalent thermal dosage was used in all groups.

**Tumor measurements and assessment.** Tumors were measured every other day with vernier calipers (Scientific Products, McGraw, IL) in two perpendicular dimensions. The tumor volume was estimated according to the formula: [(length) × (width)<sup>2</sup>] / 2 (15). Animals were followed until a quintupling of tumor volume because tumor bulk generally interfered with hind limb mobility of the animal beyond the tumor quintupling size.

Response to treatment was assessed by growth delay and regressions. Growth delay (in days) was defined as the difference in tumor quintupling time in treated versus control animals. Regressions were defined as the number of tumors whose volume decreased over two consecutive measurements (9).

**Immunohistochemical staining for proliferation marker Ki-67.** Melanoma xenografts were taken from the animals (two rats per group) at 4 and 48 hours following isolated limb infusion with 10% DMSO (in PBS) only, 10% DMSO plus hyperthermia, temozolomide (350 mg/kg) only, and temozolomide (350 mg/kg) plus hyperthermia. Tumors were fixed in 10% formalin (pH 6.9-7.1), paraffin-embedded, and 0.5 μm sections were placed on glass slides.

After epitope retrieval, sections were washed in PBS and endogenous peroxidase activity was blocked with 0.3% H<sub>2</sub>O<sub>2</sub>. Sections were then incubated with the primary anti-Ki-67 antibody (clone 7B11, dilute 1:50, Zymed Laboratories, San Francisco, CA), followed by incubating with secondary biotinylated antibody and avidin-biotin peroxidase complex (16). Reaction products were revealed with 3,3'-diaminobenzidine and sections were counterstained with Harris' hematoxylin.

Four high-powered fields (10 × 40) of tumor areas with the highest density of positive nuclear staining were chosen from each slide. The proliferative index was then determined by relating the total number of positive cells to the total number of cells in all four fields. All samples were scored independently by two observers.

**Histopathologic study of apoptosis.** Melanoma xenografts were taken from the animals (two rats per group) 48 hours following isolated limb infusion with 10% DMSO (in PBS) only, 10% DMSO plus hyperthermia, temozolomide (350 mg/kg) only, and temozolomide (350 mg/kg) plus hyperthermia. Tumors were fixed in 10% buffered formalin (pH 6.9-7.1) and were paraffin-embedded in 0.5 μm slices.

Sections were first deparaffinized and hydrated through xylene and graded ethanol series. Specimens were then permeabilized by 20-minute incubation with 20 μg/mL proteinase K in 10 mmol/L Tris (pH 8). Endogenous peroxidase was inactivated by incubating sections with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 5 minutes. Apoptotic nuclei were detected by fragment end labeling of DNA using Klenow FragEL kit (Oncogene, San Diego, CA; ref. 17). Briefly, slides were first rehydrated in PBS and then permeabilized with 20 μg/mL proteinase K in 10 mmol/L Tris (pH 8). Endogenous peroxidase was then inactivated using 3% H<sub>2</sub>O<sub>2</sub> in

methanol. Cells were then equilibrated in Klenow Equilibration Buffer followed by an incubation with Klenow Labeling Reaction mixture at 37°C for 1.5 hours in a humidified atmosphere (18). The labeled ends of DNA fragments were detected with 3,3'-diaminobenzidine. Slides were then counterstained with methyl green.

**Cytochemical staining for apoptosis.** The exponentially growing DM6 cells were incubated with 1 mmol/L temozolomide at 25°C, 37°C, or 41°C for 1 hour and then returned to a 37°C incubator for 48 hours. Heating of the cells was achieved by immersing the flasks in a preheated water bath. The three temperature conditions were chosen to simulate the three possible thermal conditions of isolated limb infusion in the rodent model and in the clinical setting. To simulate clinical isolated limb infusion, the drug concentration is ~10 times the published plasma level of temozolomide in patients treated with a systemic therapy. Temozolomide was not removed after application as the half-life of temozolomide and its active metabolite, 5-(3-methyltriazen-1-yl)imidazole-4-carboximide, is ~2 hours (19). After 48 hours, cells were trypsinized and collected together with the cells floating in the medium. The cells were washed in PBS, fixed in 4% formaldehyde, then resuspended in 80% ethanol, and stored at 4°C for 24 hours.

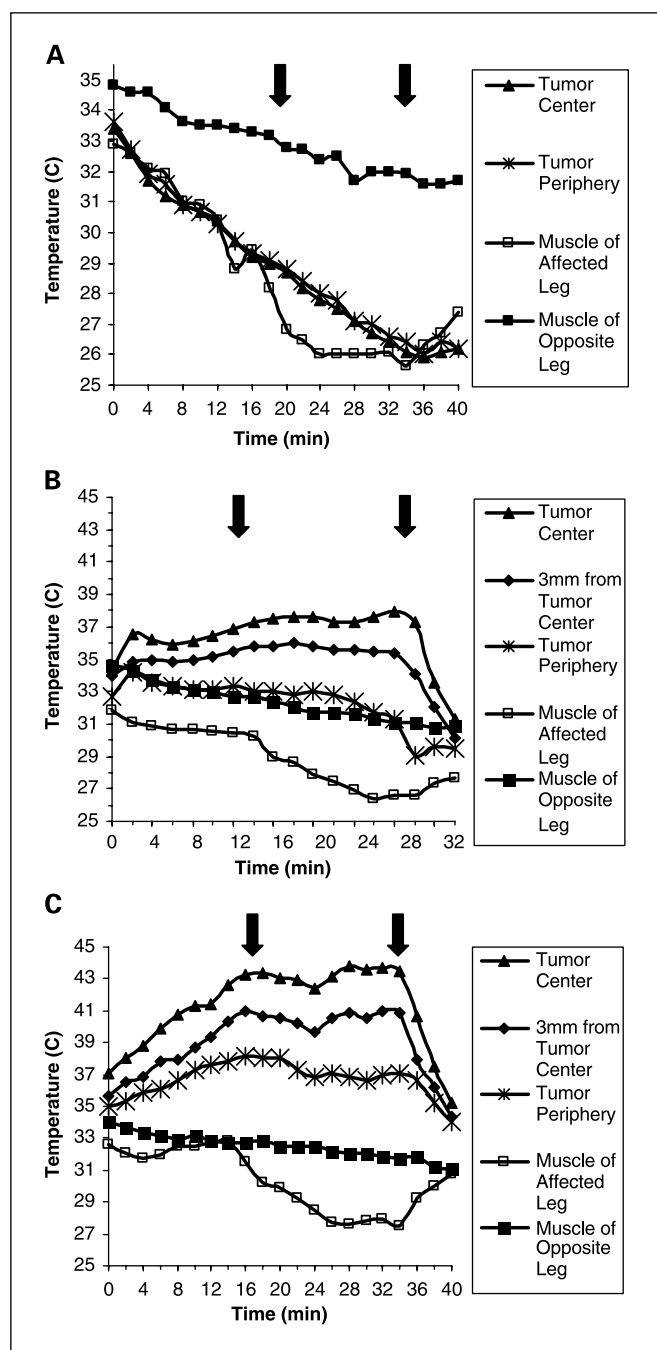
The fixed cells were immobilized onto glass slides by using Cytospin (Shandon). Apoptotic nuclei were detected by fragment end labeling of DNA using Klenow FragEL kit (Oncogene) as described above (17).

## Results

**Temperature profile during isolated limb infusion.** Figure 2 shows a representative temperature profile during surgery under different thermal conditions. Without the application of the heating device (Fig. 2A), the temperature of the operative leg fell to hypothermic conditions (26-27°C) during the limb infusion. When the heating surface of the catheter, located in the approximate center of the tumor, was maintained at 37.5°C, normothermic conditions were maintained (Fig. 2B). Heating the catheter surface to 43°C produced hyperthermic conditions (Fig. 2C). Because heat was applied locally to the tumor, the temperature of the tissues beyond the surface of the tumor was not affected by the heating device as shown by the hypothermic temperature (26-27°C) of the muscle of the operative leg during the infusion (Fig. 2B and C). The heat dosages were consistent for all animals, as average total CEM43°C values were not significantly different between the treatment groups subjected to hyperthermia (Table 1).

**Therapeutic studies.** The tumor growth was marginally affected by isolated limb infusion with 10% DMSO plus normothermia or hyperthermia, with tumor growth delay of 0.4 and 1.8 days, respectively (Fig. 3; Table 2). These values did not reach a statistical significance ( $P = 0.26$  and  $0.11$ ). A low-dose (350 mg/kg) temozolomide treatment alone produced no tumor growth delay compared with the control values (Fig. 3; Table 2). Isolated limb infusion under normothermic conditions with low-dose temozolomide also had little effect on the tumor growth with only a small increase of 0.8 days in tumor growth delay. In contrast, the simultaneous application of hyperthermia during a low-dose temozolomide infusion led four of six treated animals to respond with marked tumor growth inhibition (Fig. 3D). This addition of hyperthermia to isolated limb infusion with low-dose temozolomide produced a statistically significant average growth delay of 10.4 days ( $P = 0.02$ ) compared with the control value as well as increased thermal enhancement ratios (Table 2).

There was no statistically significant difference in the number of tumor regressions in rats treated with temozolomide



**Fig. 2.** A representative temperature profile during isolated limb infusion without the addition of heat (A) or under controlled normothermia (B) or hyperthermia (C). Arrows, start and stop of the infusion. Temperature readings were recorded every 2 minutes from the onset of the surgery (defined as the time of the first incision) until the conclusion of the surgery (defined as the time of the wound closure).

compared with those in the control group (Table 2). Two of six animals in the control group also responded with early regression after treatment. This result is likely an artifact of the design of this study, which required puncturing the tumor center using a 14 G catheter for the heat application (Fig. 1). Although there was no significant difference in the overall number of tumor regressions between each group, the rats treated with low-dose temozolomide in combination with hyperthermia maintained regression longer than those in the control group (Fig. 3).

There were no significant differences in the mean nadir weight loss of rats treated with temozolomide compared with those in the control group (Table 2). The use of heat produced no noticeable local limb toxicity to animals treated with a low-dose temozolomide (350 mg/kg). These animals showed a full recovery of the hind limb function within 2 weeks after surgery. However, four of five animals treated with the high-dose (1,500 mg/kg) temozolomide in combination with normothermia showed severe limb necrosis and autoamputation by the 3rd day after surgery (Table 2). Similarly, three of six animals treated with high-dose (1,500 mg/kg) temozolomide in combination with hyperthermia showed autoamputation (Table 2). In addition, one of three surviving animals treated with high-dose temozolomide plus hyperthermia had severe edema and failed to recover its hind limb function after surgery. These animals were sacrificed according to the animal care protocol and were excluded from the survival study. None of the animals treated with high-dose temozolomide in the absence of heat showed evidence of limb toxicity after surgery.

**Tumor cell proliferation following isolated limb infusion.** To define the growth fraction in DM6 xenografts after various treatment regimens, we examined the Ki-67 proliferative indices 4 and 48 hours after isolated limb infusion. At the 4-hour interval, the proliferative index was similar among animals treated with 10% DMSO, 10% DMSO plus hyperthermia, or 350 mg/kg temozolomide (index =  $0.84 \pm 0.03$ ,  $0.82 \pm 0.03$ , or  $0.87 \pm 0.02$ , respectively). There was a slight decrease in the proliferative index of animal treated with 350 mg/kg temozolomide and hyperthermia (index =  $0.76 \pm 0.04$ ) but this was not statistically significant when compared with the control ( $P = 0.15$ ).

By 48 hours postsurgery, the tumors treated with 10% DMSO plus hyperthermia and temozolomide plus hyperthermia both showed a central area of necrosis with viable cells in the periphery (data not shown). These peripheral areas of the tumor had the highest density of positive nuclear staining for Ki-67 and, therefore, were chosen from each slide for analysis (Fig. 4). As shown in Fig. 4E, the proliferative index 48 hours after isolated limb infusion remained high in animals treated with 10% DMSO (index =  $0.92 \pm 0.02$ ), and in animals treated with 10% DMSO plus hyperthermia or with temozolomide alone (index =  $0.90 \pm 0.02$  or  $0.84 \pm 0.01$ , respectively). There was no statistical difference between these groups ( $P = 0.96$ ,  $0.15$ ). In contrast, 48 hours after isolated limb infusion, there was a statistically significant decrease in proliferative index in tumors treated with temozolomide plus hyperthermia (index =  $0.73 \pm 0.02$ ,  $P = 0.001$ ; Fig. 4D and E).

**Apoptosis following temozolomide treatment.** To determine the effect of heat on temozolomide-treated melanoma xenograft, we did a histologic staining of fragmented DNA by end labeling on tumors taken 48 hours after isolated limb infusion treatments. The amount of apoptosis in the tumors treated with 10% DMSO or temozolomide alone remained minimal (Fig. 5A and C). The tumors treated with 10% DMSO plus hyperthermia and temozolomide plus hyperthermia both showed a central area of necrosis with viable cells in the periphery by 48 hours postsurgery (data not shown). Within this viable periphery of the tumor, the amount of apoptosis was minimal in tumors treated with 10% DMSO plus hyperthermia (Fig. 5B). In contrast, tumors treated with temozolomide and hyperthermia showed more numerous apoptotic cells within the viable periphery 48 hours after isolated limb infusion (Fig. 5D).



**Table 1.** Average tumor temperature and thermal dosimetry during normothermic and hyperthermic isolated limb infusion

	Treatment group				
	10% DMSO + NT	350 mg/kg TMZ + NT	10% DMSO + HT	350 mg/kg TMZ + HT	1,500 mg/kg TMZ + HT
1 mm from tumor center*	36.0 ± 1.1	36.7 ± 0.6	41.3 ± 1.0	41.7 ± 1.0	41.0 ± 0.8
3 mm from tumor center	34.5 ± 0.9	35.2 ± 0.4	36.9 ± 1.2	38.8 ± 0.9	39.4 ± 1.7
Tumor periphery	33.5 ± 0.8	33.4 ± 1.1	34.6 ± 1.4	36.5 ± 1.3	36.3 ± 1.4
Duration of heat†	40.0 ± 5.4	40.5 ± 7.2	38.7 ± 4.1	38.7 ± 3.3	44.0 ± 10.3
Average total CEM43°C‡	—	—	35.3	39.7 (0.52)	47.2 (0.72)

Abbreviations: TMZ, temozolomide; NT, normothermia; HT, hyperthermia.

\*Average tumor temperature ± SD is displayed for each treatment group ( $n = 6$ ).

†Average number of minutes the tumor was heated ± SD for each treatment group.

‡Average total CEM43°C for each treatment group where hyperthermia was applied.  $P$  values comparing CEM43°C of each hyperthermic treatment group to 10% DMSO + hyperthermia group is shown in parenthesis.

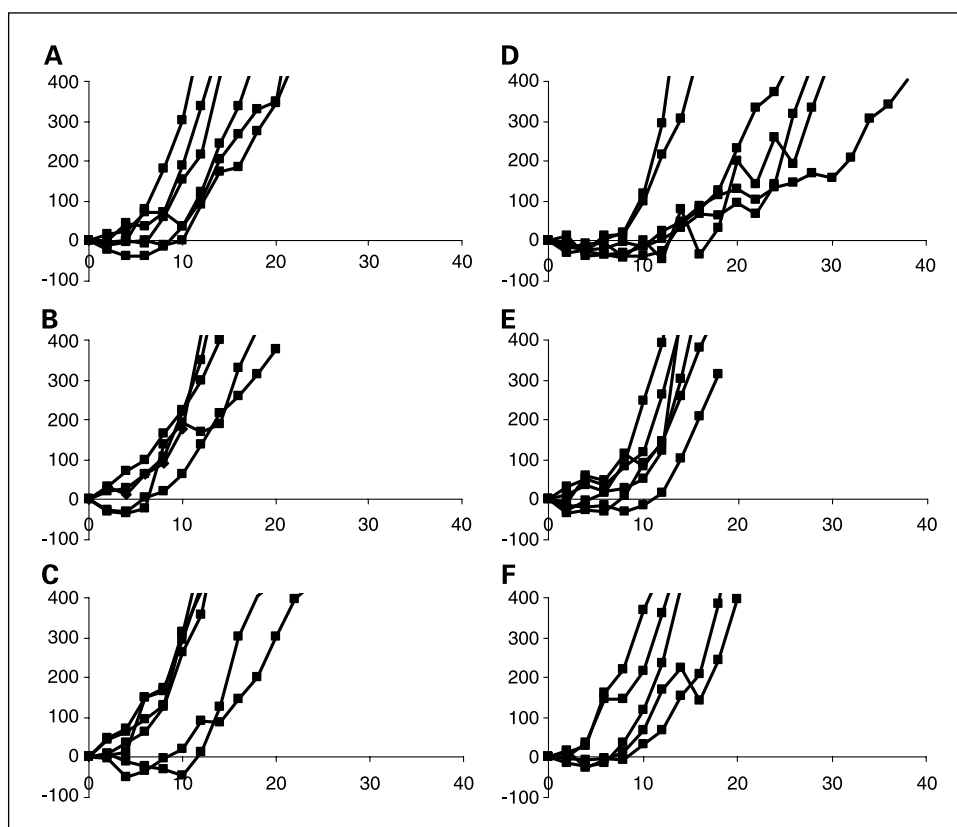
To better show the proapoptotic effect of heat on temozolomide-treated melanoma cells, we also did a cytochemical staining of fragmented DNA by end labeling on DM6 cells that were subjected to uniform heating temperatures. The percentage of apoptotic cells was highest in those cells treated with temozolomide plus mild hyperthermia at 41°C (Fig. 6). The cells treated with temozolomide at 25°C or 37°C showed a slight induction of apoptosis 48 hours after temozolomide treatment (Fig. 6D and E). The cells treated with heat alone showed few apoptotic nuclei (Fig. 6C). The cells treated with

temozolomide at 41°C were strongly driven into programmed cell death with a greater percentage of apoptotic nuclei per slide (Fig. 6F).

## Discussion

Historically, despite appropriate initial therapy, ~2% to 10% of extremity melanoma lesions (1,000-5,000 cases/y) would recur in the extremity as in-transit metastasis representing melanoma tumor deposits in the dermal or s.c. lymphatic

**Fig. 3.** Tumor growth curves of DM6 xenografts following isolated limb infusion with 10% DMSO solution under hyperthermic (A) or normothermic (B) condition or following isolated limb infusion without heat (C). Tumor growth following isolated limb infusion with 350 mg/kg temozolomide with heat under hyperthermic (D) or normothermic (E) conditions or without heat (F). Each curve represents the tumor response of an individual animal. Y axis, viable tumor volume relative to starting tumor volume (in percentage); X axis, number of days postsurgery. Animals were followed until a quintupling of tumor volume (equivalent to reaching the maximum Y-axis value of 400%).



**Table 2.** Effect of regional temozolomide treatment via isolated limb infusion with or without localized mild hyperthermia on DM6 human melanoma xenografts grown in athymic rats

Heat	TMZ (mg/kg)	Initial tumor size* (cm <sup>3</sup> )	Tumor-quintupling time <sup>†</sup> (d)	Tumor growth delay <sup>‡</sup> (d)	TER <sup>§</sup>	Regression <sup>  </sup>	Weight loss <sup>¶</sup> (%)	Toxicity-related limb necrosis
None	0**	1.4 ± 0.1	14.2 ± 1.9	—	—	2/6	-9.8 ± 1.0	0/6
NT	0**	1.2 ± 0.1	14.6 ± 1.4	0.4 (0.26)	—	2/6	-7.9 ± 2.3	0/6
HT	0**	1.1 ± 0.2	16.0 ± 1.7	1.8 (0.11)	—	2/6	-8.5 ± 1.6	0/6
None	350	1.3 ± 0.1	14.0 ± 1.7	-0.2 (0.07)	—	2/6	-11.9 ± 1.6	0/6
NT	350	1.3 ± 0.1	15.1 ± 1.2	0.8 (0.08)	4	1/6	-11.4 ± 1.9	0/6
HT	350	1.2 ± 0.1	24.6 ± 3.8	10.4 (0.02)	52	3/6	-9.9 ± 2.7	0/6
None	1,500	1.4 ± 0.1	17.4 ± 3.8	3.2 (0.39)	—	2/6	-6.9 ± 1.2	0/6
NT	1,500	1.3	13.6	-0.6 (0.62)	0.2	0/1	-10.4	4/5
HT	1,500	1.2	25.4	11.2 (0.09)	3.5	0/2	-12.6	4/6

\*Tumor volume (on day 0 before treatment) was estimated according to the following formula: [(length) × (width)<sup>2</sup>] / 2 (mean ± SE).

<sup>†</sup>Time taken for individual tumor to reach a volume of five times its untreated volume on day 0 (mean ± SE).

<sup>‡</sup>The difference between the mean time required for tumors in treated and control rats to reach a volume of five times their untreated volume on day 1. Number in parenthesis, *P* value compared with the control (10% DMSO group without heat) obtained using Wilcoxon rank sum test.

<sup>§</sup>Thermal enhancement ratio = growth delay of temozolomide + heat group / growth delay of temozolomide without heat group.

<sup>||</sup>Number of tumors whose volume decreased over two consecutive measurements.

<sup>¶</sup>Mean body weight nadir (±SE) as a percentage.

\*\*DMSO (10%) in PBS was used as the infusate for isolated limb infusion when no temozolomide was used.

vessels (20). In a more recent study, using a prospective database of patients undergoing lymphatic mapping and sentinel lymph node biopsy, ~6.5% of patients with extremity melanoma developed in-transit recurrence at a mean follow-up of only 3.5 years (21). Interestingly, in 71% of these patients, the in-transit disease was the only site of recurrence. Tumor recurring in this fashion usually appears as multiple nodules throughout the extremity. Because this pattern of recurrence represents multifocal involvement of the extremity lymphatic system, local excision of these in-transit lesions is frequently followed by rapid recurrence. However, amputation of the extremity has been associated with long-term survival in ~25% to 30% of patients in several series, one of which had 15-year follow-up (22–24). Whereas few would advocate amputation as the primary treatment modality for this group of patients, these studies do show that with appropriate aggressive therapy encompassing all locoregional disease, long-term disease control and survival is possible for a subset of these patients in the absence of systemic treatment.

Although most patients with advanced regional disease do eventually succumb to systemic disease, systemic therapies in melanoma have response rates of only 15% with few complete responders. Regional therapy using melphalan in either the form of hyperthermic isolated limb perfusion or infusion delivers a drug dose to the tumor that is 10 times greater than can be achieved with systemic therapy and has shown the ability to achieve long-term complete responses. A number of single institution studies (3, 25–29) have shown response rates in the 80% range with 40% to 60% being complete responders. Although studies that looked at the prophylactic use of regional therapy in early melanoma have failed to show a survival advantage to this form of therapy, these studies were underpowered with regard to the number of patients in whom this treatment would benefit (i.e., those who would develop recurrent regional disease) to truly answer this question. However, all these studies did show a lower incidence of local

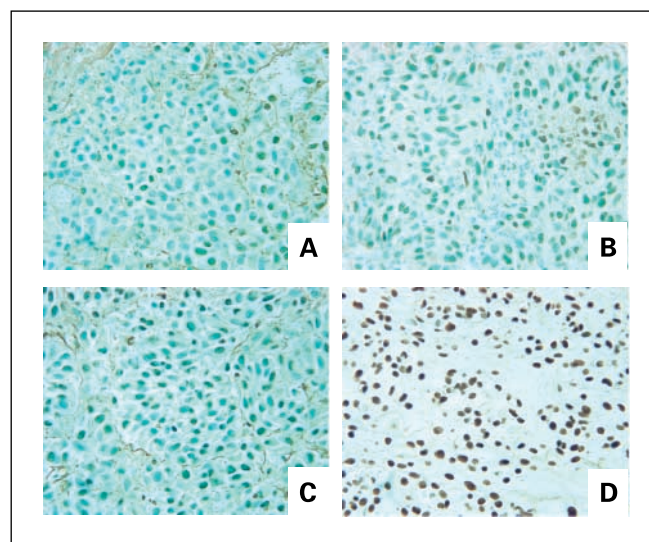
recurrence and in-transit disease in those receiving regional therapy.

Despite these encouraging results, the majority of patients who undergo regional infusional therapy still have persistent or locally recurrent disease in the absence of distant metastatic disease. In addition, ~1% to 3% of patients suffered complications leading to amputation, 10% to 15% developed compartment syndrome, 30% to 40% developed lymphedema, and 5% to 8% develop long-term neuropathy (30, 31). The lack of response durability, the large number of partial responders, and the moderate limb toxicity associated with melphalan-based treatment has led to the exploration of novel approaches to optimizing regional therapy (32–34). A recently completed randomized prospective phase III trial run by American College of Surgeons Oncology Group that compared melphalan alone to melphalan plus tumor necrosis factor was stopped at its interim analysis by the data safety monitoring committee because no advantage to tumor necrosis factor was identified. In addition, the single institution response rates of 80% using melphalan were not reproduced, with overall response rates in the American College of Surgeons Oncology Group trial being ~70% in both arms with only 30% of patients in each arm exhibiting a complete response. The disappointing results of this trial has left open the need to develop more effective, durable, and less toxic regional treatment strategies.

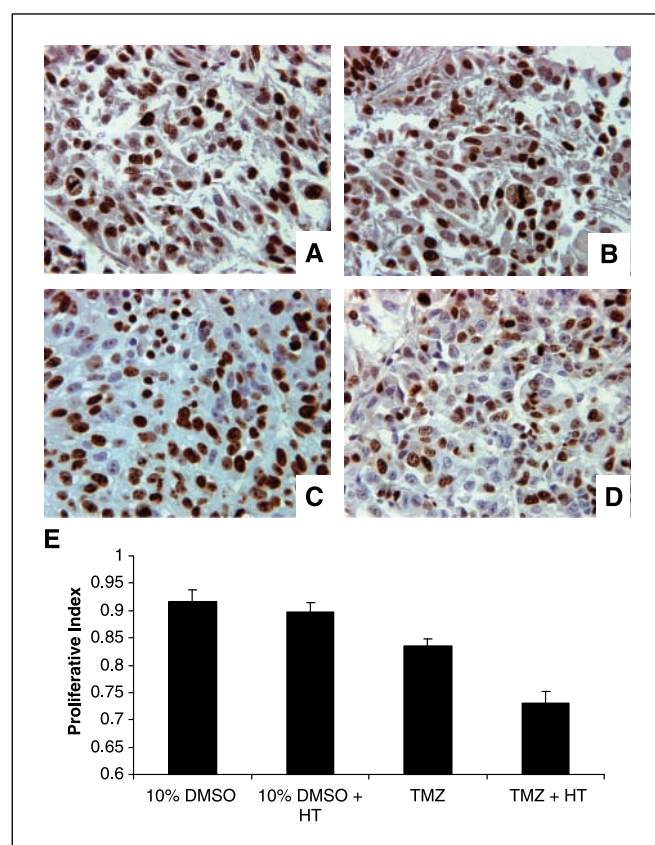
Interestingly, our recent preclinical studies have shown that regional temozolomide therapy was more effective than either systemic temozolomide or regional melphalan in an animal model of advanced extremity melanoma (5). Temozolomide is an alkylating agent that has the best single-agent activity against metastatic melanoma when used systemically (10). However, the clinical response rate is only 15% to 20% and few patients achieve a complete response. Whereas regional therapy with temozolomide is expected to be more effective because of the ability to deliver a higher dosage to the tumor while avoiding systemic toxic effects, temozolomide has not yet been used as a

regional chemotherapy agent in humans due to its unavailability as an i.v. formulation. Preliminary clinical studies are currently under way with the newly developed i.v. formulation of temozolomide and a phase I study using temozolomide intra-arterially is currently in development. Therefore, the aim of this study was to explore the potential benefits in applying heat with the concomitant administration of temozolomide via the isolated limb infusion technique in the treatment of in-transit melanoma. Because the application of heat can improve the therapeutic index of many alkylating agents, we wanted to determine whether hyperthermia could augment temozolomide therapeutic efficacy as we prepare for phase I clinical trials.

Without the addition of heat, the temperatures within the operative leg fell to hypothermic conditions during isolated limb infusion (Fig. 2A). Using a unique method of heat application, we were able to apply localized heating to the tumor in a normothermic or hyperthermic temperature range in a controlled and reproducible manner (Fig. 2B and C). Viglianti et al. (35) has shown that the heating device we used for these studies consistently yields a thermal profile that fits to



**Fig. 5.** A representative stain for apoptosis by *in situ* immunohistochemical labeling of exposed ends of fragmented DNA on DM6 xenografts 48 hours after isolated limb infusion with 10% DMSO (A), 10% DMSO + hyperthermia (B), 350 mg/kg temozolomide (C), or 350 mg/kg temozolomide + hyperthermia (D). Apoptotic cells (brown nuclear staining) are much more numerous in the xenograft treated with temozolomide + hyperthermia compared with the xenografts of other treatment groups, (magnification,  $10 \times 40$ ).



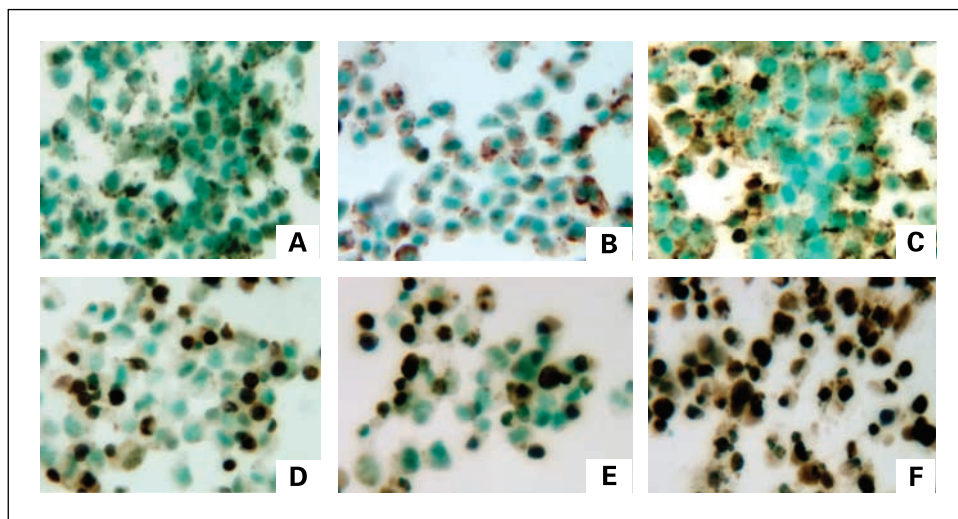
**Fig. 4.** A representative immunohistochemical stain for proliferation marker Ki-67 on DM6 xenograft 48 hours after isolated limb infusion with 10% DMSO (A), 10% DMSO + hyperthermia (B), 350 mg/kg temozolomide (C), or 350 mg/kg temozolomide + hyperthermia (D). Treatment with temozolomide + hyperthermia led to a marked decrease in number of cells positive for Ki-67 proliferation marker (dark brown nuclear staining). Average Ki-67 proliferative indices of the melanoma xenograft 48 hours after various isolated limb infusion treatments (E). Proliferative index was determined by relating total number of positively staining cells to total number of cells in four high-powered fields ( $10 \times 40$ ). Columns, average proliferative index for two animals in each group; bars, SE. There was a statistically significant difference ( $P = 0.001$ ) between the proliferative indices of the group treated with temozolomide (TMZ) + hyperthermia (HT) and that of the group treated with 10% DMSO.

the radial one-dimensional steady-state heat transfer equation, with the hottest temperature near the catheter and radially decreasing temperature toward the tumor periphery. The consistency in the administration of hyperthermic doses to the tumor is important because variations in tumor temperature as small as  $2^{\circ}\text{C}$  maintained for over 30 minutes can result in vastly different fractions of tumor cell killing (14, 36). Various authors have also shown that the thermal enhancement ratios are strongly temperature dependent for many alkylating drugs, such as mitomycin C and melphalan (37, 38).

The thermal enhancement of temozolomide activity was striking in this nude rat model, as shown by the substantial tumor growth delay as the temperature was increased from hypothermic ( $27.5\text{--}28.7^{\circ}\text{C}$ ) to normothermic ( $34.1\text{--}37.3^{\circ}\text{C}$ ) to hyperthermic ( $36.8\text{--}42.5^{\circ}\text{C}$ ) conditions during isolated limb infusion with temozolomide (Fig. 3; Table 2). These results strongly parallel the *in vivo* studies by Takemoto et al. (39), which showed that the alkylating agents are most effective at elevated temperatures.

The combination of hyperthermia and regional temozolomide treatment also resulted in decreased tumor proliferation, as shown by Ki-67 expression 48 hours following isolated limb infusion (Fig. 4). Ki-67 is a nuclear antigen that is present in all phases of cell cycle, except  $G_0$  and early  $G_1$ . Moretti et al. (40) found that Ki-67 proliferative index is an indicator of poor prognosis and a possible predictor of metastasis in primary melanomas. Plaat et al. (41) reported that the posttreatment proliferative index in soft tissue sarcomas from patients undergoing hyperthermic isolated limb perfusion with melphalan correlated with overall survival following treatment. Our study showed that the addition of heat to temozolomide treatment yielded the smallest proliferative index 48 hours after isolated limb infusion, indicating that heat interacts synergistically with temozolomide to reduce the proliferative fraction of the tumor.





**Fig. 6.** Apoptosis of DM6 cells determined by *in situ* fragment end labeling of DNA. Cells were incubated at 25°C (A), 37°C (B), or 41°C (C) for 1 hour and then returned to 37°C incubator for 48 hours. Cells were treated at 25°C (D), 37°C (E), or 41°C (F) for 1 hour in a medium containing 1 mmol/L temozolomide and then returned to 37°C incubator for 48 hours. Cells were harvested and fixed on to slides using Cytospin. Apoptotic cells are much more numerous in the cell population treated with temozolomide at 41°C (F) than in the other cells, (magnification, 10 × 40).

The addition of heat to isolated limb infusion with temozolomide also seemed to increase the apoptotic activity (detected using a tissue staining of fragmented DNA by end labeling) in the tumor 48 hours postprocedure (Fig. 5). The tumors treated with hyperthermia consistently showed a central area of necrosis with the viable cells in the periphery by 48 hours postsurgery. The application of hyperthermia alone seemed to have little effect on the viable tumor cells in the periphery (Fig. 5B). In contrast, numerous apoptotic bodies were found in the periphery of the tumor treated with temozolomide plus hyperthermia (Fig. 5D). This was likely a result of the radial temperature gradient across the xenograft created by the heating device. Whereas the elevated temperature of 43°C near the surface of the catheter resulted in cell necrosis, milder hyperthermic temperatures (below 43°C) may be inducing apoptosis in the temozolomide-treated melanoma cells. Therefore, we also assessed for apoptotic activity *in vitro* where we were able to apply uniform heating to all cells at mild hyperthermic temperature of 41°C. This temperature is representative of the average temperature during the hyperthermic isolated limb infusion in the animal model and in the clinical setting.

The addition of heat to temozolomide treatment resulted in a marked increase in the percentage of cell death by 48 hours posttreatment *in vitro* (Fig. 6). Possible mechanisms of this thermal chemosensitization include increasing the reaction rate and decreasing the ability of DNA repair. The rate constant of a chemical reaction that leads to cell death increases with escalation in temperature (42). Several authors reported an enhanced formation of DNA cross-links following treatment with alkylating agents at elevated temperatures (43, 44). Hettinga et al. (45) also reported that cellular ability to repair

DNA adducts formed by platinum-based alkylating agents were impaired at elevated temperatures. Further work investigating the effect of various thermal dosages on the DNA methylation following temozolomide treatment would help clarify the mechanism of the chemosensitization by heat.

Although the simultaneous application of heat and regional temozolomide resulted in the greatest therapeutic benefit with increased tumor apoptosis, there was also an evidence of increased toxicity. When a high-dose (1,500 mg/kg) temozolomide infusion was combined with heat, four of five animals treated with normothermia and four of six animals treated with hyperthermia showed severe limb necrosis and autoamputation. When the high-dose temozolomide infusion was used without heat, none of the animals showed similar levels of toxicity. This suggests that the addition of heat narrows the therapeutic index of the alkylating agent. However, when hyperthermia was applied in conjunction with a low-dose temozolomide, no apparent toxicity was observed in the limbs of the animal while markedly improving the therapeutic index of temozolomide activity. These results indicate that small increments of heat escalation in strict and reproducible dosages of hyperthermia will be necessary to minimize the potential toxicity of this treatment in the design of phase I clinical trials.

In summary, this study shows strong synergistic antitumor effects when hyperthermia is combined with temozolomide regional treatment. These results also suggest for a heating method that selectively heats the tumor rather than the whole limb to reduce the limb toxicity while maximizing the therapeutic benefit. These findings may serve as important guidelines for further development of clinical trials of isolated limb perfusion and infusion using temozolomide in conjunction with hyperthermia.

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