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Thermochemotherapy *In Vivo* of a C3H Mouse Mammary Carcinoma: Single Fraction Heat and Drug Treatment

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Abstract—*The interaction between water bath hyperthermia* (43.5°C) *and six cancer chemotherapeutic agents* in vivo *was studied in a transplantable C3H mouse mammary carcinoma grown s.c. in the feet of C3D2F1/Bom mice. Due to differences in tumour regrowth rate between treatment groups, both tumour growth time (TGT) and specific growth delay (SGD) were used as effect parameters. The largest tumour response was observed when the drug was given* 15 *min prior to heat—this timing was used for dose–effect experiments. Enhancement ratios were the ratios of slopes of dose–effect curves subjected to linear regression analysis. The drug enhancement ratio (DER) was not significantly larger than* 1.0 *for LD* 1% *of adriamycin, 5-fluorouracil, methotrexate and vincristine. For cyclophosphamide (CTX) and mitomycin C (MMC) both DER and TER (thermal enhancement ratio) were significantly larger than* 1.0. *The TGT ratios (SGD ratios in parentheses) were: DER (LD* 1%): *CTX* 1.4 ± 0.1 (2.1 ± 0.1), *MMC* 1.3 ± 0.1 (1.4 ± 0.1); *TER* (43.5°C 30 *min*): *CTX* 1.6 ± 0.1 (2.7 ± 0.2), *MMC* 2.8 ± 0.5 (3.3 ± 0.7). *The data support the choice of CTX and MMC in preference to the other drugs investigated for clinical thermochemotherapy studies.*

INTRODUCTION

THE THERAPEUTIC potential of chemotherapy and hyperthermia given separately for treatment of malignant tumours has obvious limitations. The doses required of either of the two treatments to obtain tumour cure may not be tolerated by normal tissues or hosts. There is potential for improvement of treatment results by the combination of heat treatment and chemotherapy, thermochemotherapy [1–5]. More experimental data on the magnitudes of reciprocal enhancements of single drugs and heat *in vivo* in tumours and normal tissues are, however, necessary in order to substantiate the scientific basis for clinical local thermochemotherapy. We have studied the interaction between heat treatment at 43.5°C and six cancer chemotherapeutic agents *in vivo* in a C3H mouse mammary carcinoma. All drugs employed are commonly used for treatment of patients with breast carcinoma. The aim of the study was to determine the optimal timing and sequence of administration of heat and drug and to determine magnitudes of drug and heat enhancement of single treatments in the tumour.

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MATERIALS AND METHODS

Tumour and mice

The animal tumour system has previously been comprehensively described [6, 7]. The tumour is a spontaneously arisen, moderately differentiated C3H/Tif mammary carcinoma. The tumour was propagated by serial transplantation. Large flank tumours were dissected under sterile conditions. Macroscopically viable tumour tissue was minced with a pair of scissors and forced through sterile needles of decreasing dimensions. Approximately 0.02 ml of tumour material was inoculated subcutaneously through a 25 gauge needle into the dorsal side of the right hind foot of female C3D2F1/Bom mice (C3H ♀ × DBA/2 ♂) weighing between 19 and 24 g. Tumour growth occured in close to 100% of inoculated feet. Tumour volume was calculated using the formula $D_1 \times D_2 \times D_3 \times \pi/6$, where

D_{1-3} are three orthogonal diameters measured with a slide gauge. All tumour measurements and treatments were performed by one person. The mice were kept three per cage at room temperature, 21–22°C. They were fed a standard pellet diet, R3 (Ewos AB, Södertälje, Sweden), and given water *ad libitum*. Single treatments with heat alone, drug alone or a combination of the two were carried out when the tumours had reached a volume of 130–200 mm^3 10–17 days after inoculation. The experiments were performed on tumour generations 6–29.

Heat treatment and thermometry

Heat treatment of tumour-bearing legs was performed using a heated water bath. Unanaesthetized mice were trapped in lucite plastic jigs, perforated to allow circulation of air. Tumour-bearing legs were fixed to an extension of the jigs by applying paper tape loosely around the legs above the tumours. Care was taken to avoid impairment of the blood circulation. The heatable water bath containing approx. 25 l, was covered by a lucite plastic plate with holes allowing immersion of the tumour-bearing leg into the water with the tumour approx. 1 cm below the water surface. The thermostatically controlled temperature in the water bath was ±0.02°C of the set temperature. In control experiments intratumoural temperatures were measured with 0.8 mm single point probes, a thermistor (Lund Science AB, Lund, Sweden) and a fluoroptic probe (Luxtron, Mountain View, California). With both types of thermometry, the core temperature of tumours measuring between approx. 100 and 300 mm^3 stabilized approx. 0.2°C below water temperature (Fig. 1). All heat treatments of tumours included in the dose–effect experiments were carried out without invasive thermometry. The stated treatment times at a tumour temperature of 43.5°C are the total times of immersion of tumour-bearing legs into heated water maintained at 43.7°C. Ambient room temperature during treatment was 21–23°C. Tumour core temperature was 25–26°C in animals kept at room temperature in the treatment position prior to immersion of the foot into water. Control measurements of rectal temperatures were performed in some mice during heat treatment. Body temperature increases of 2–3°C were recorded during heat treatments.

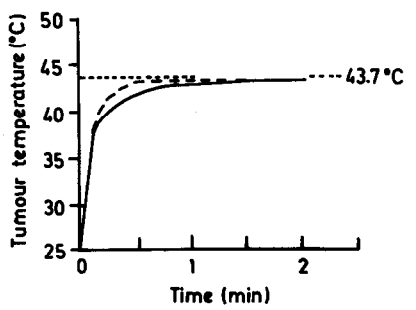

Fig. 1. Tumour core temperature measured with a fluoroptic probe and a thermistor probe (broken line) in a 155 mm^3 tumour during heat treatment in a water bath adjusted to 43.7°C (dotted line). The measured tumour temperatures stabilize approx. 0.2°C below the water bath temperature.

Drug treatment

The drugs investigated were cyclophosphamide (CTX) (Lääke Farmos-Farmitalia Carlo Erba), doxorubicin (ADR) (Farmitalia Carlo Erba), 5-fluorouracil (5FU) (F. Hoffmann-La Roche), methotrexate (MTX) (Nycomed), mitomycin C (MMC) (Bristol-Myers) and vincristine (VCR) (Eli Lilly & Co.). For each drug the highest dose utilized was the maximum tolerated dose (MTD)—the drug dose that kills approx. 1% of animals within 150 days (Table 1). This dose was estimated in separate experiments for MTX and VCR. The other MTDs were taken from Ref. [8] in which estimations of MTD were made in the same mouse strain. All drugs were dissolved in sterile distilled water. For ADR, CTX and MMC stem samples of diluted drugs were divided in 10 ml vials and frozen at −20°C [9]. The drugs were given intraperitoneally (i.p.) at a constant volume of 0.02 ml/g body wt.

*Table 1. Maximum tolerated drug doses (MTD)**

Drug	Abbreviation	MTD (mg/kg)
Cyclophosphamide	CTX	100
Doxorubicin	ADR	8
5-Fluorouracil	5FU	150
Methotrexate	MTX	150
Mitomycin C	MMC	3
Vincristine	VCR	2

*MTD equivalent to $LD_{01/150\ days}$.

Evaluation of results

Based on measurements of tumour volume at least 3–4 times a week, individual growth curves were constructed for each tumour. The time to reach a tumour volume of 5 times the volume at treatment was used as endpoint, and recorded as the tumour growth time (TGT). The growth rate during the last 3–4 days before reaching the endpoint was recorded as the tumour volume doubling time (DT). As a means of compensating for differences in DTs between treatment groups, the data were also analysed using specific growth delay (SGD) as the effect parameter.

Specific growth delay was defined as

$$SGD = \frac{TGT_{treated} - TGT_{untreated}}{DT_{treated}}$$

where $TGT_{untreated}$ is the mean TGT in 95 untreated controls.

Dose–effect curves were constructed for TGT and SGD vs. (1) heat dose (heating time at 43.5°C), for heat alone and for MTD of each of the six drugs given 15 min prior to heat treatment; (2) drug dose, for drug alone (CTX and MMC) and for each of the two drugs given 15 min prior to 43.5°C 30 min. Approximately 16 animals were included in each treatment group. For heat alone 40–50 animals were included at each dose point. The drug enhancement ratio (DER) is the ratio of the slopes of a drug + heat curve to the slope of the curve for heat alone. Thermal enhancement ratio (TER) is the ratio of slopes of a curve for drug +43.5°C 30 min to the slope of the curve for the corresponding drug alone. All enhancement ratios were determined both with TGT and SGD as effect parameter. Significance levels given by *t*-tests are based on slopes ± S.D. obtained from linear regression analysis, indicating at which level a ratio is different from 1.0.

The experimental protocol was approved by the Experimental Animal Board.

RESULTS

Tumour growth curves in groups of mice given MTD of each of the six drugs are presented in Fig. 2. Tumour growth curves in mice given single doses of CTX alone are presented in Fig. 3. Examples of tumour growth curves after different single fraction treatments—heat alone, CTX alone and CTX given 15 min prior to heat treatment—are presented in Fig. 4.

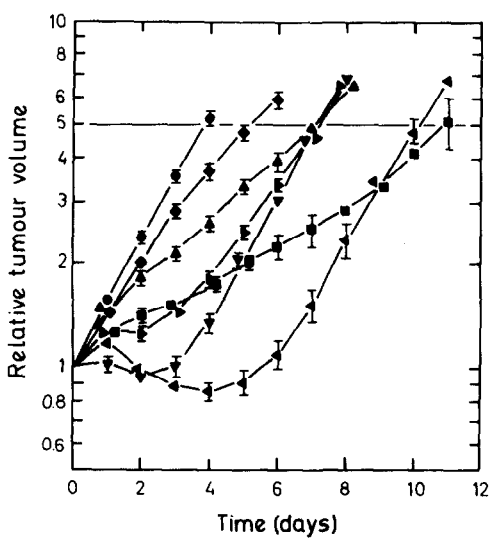

Fig. 2. Tumour growth curves in groups of mice given single drug treatment alone with maximum tolerated drug doses. Untreated controls ●, MMC ◆, ADR ▲, 5FU ▶, MTX ▼, VCR ◀ and CTX ■. The data points are the mean relative volumes and S.E. based on mainly daily measurements of tumour volumes in all contributing mice.

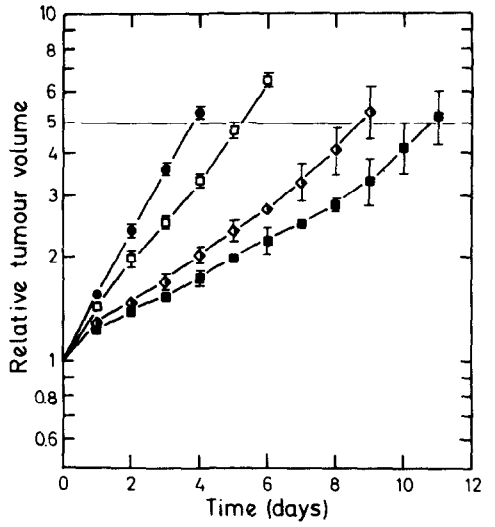

Fig. 3. Tumour growth curves in groups of mice given single doses of CTX. Untreated controls ●, 33 mg/kg □, 67 mg/kg ◆ and 100 mg/kg ■. The data points are the mean relative volumes and S.E. based on mainly daily measurements of tumour volumes in all contributing mice.

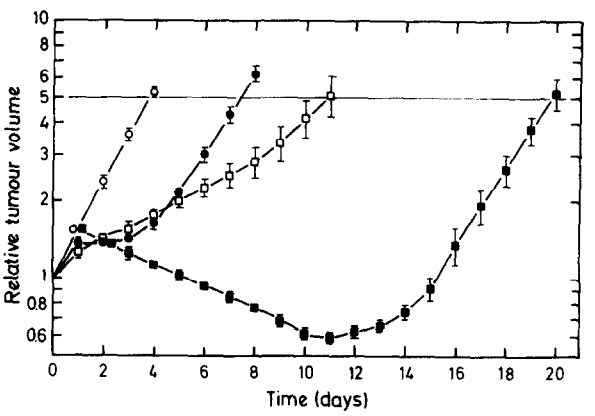

Fig. 4. Examples of tumour growth curves in groups of mice given different types of single fraction treatments. Untreated controls ○, 43.5°C, 30 min ●, CTX 100 mg/kg □ and CTX 100 mg/kg 15 min prior to 43.5°C, 30 min ■. The data points are the mean relative volumes and S.E. based on mainly daily measurement of tumour volumes in all contributing mice.

There were variations in DTs not only between treatment groups given MTD of different single drugs alone (Fig. 2), but the increases in TGTs observed after increasing doses of single drug alone were also partly due to increases in DTs for both CTX (Fig. 3) and MMC. When single drug was given in combination with the shortest heat treatment (10 min), the DTs were grossly unchanged compared to the DTs after the same drug given alone. When drug treatment was combined with a longer heat treatment (≥30 min), the observed DTs were comparable to the DTs observed after the same heat treatment given alone (Fig. 4). The variation in DT between treatment groups is accounted for by the parallel use of both TGT and SGD as effect parameters resulting in double sets of enhancement ratios. In the dose–effect curves presented, TGT is used as the effect parameter.

In Fig. 5A–D the respective dose–effect curves for the maximum tolerated doses of ADR, 5FU, MTX and VCR given 15 min prior to heat treatment at 43.5°C are presented in comparison with the dose–effect curve for heat alone. The corresponding slopes and DERs are given in Table 2. The DERs are not significantly larger than 1.0 for any of the four drugs. With SGD the DERs for MTX and VCR were significantly smaller than 1.0.

The results of timing and sequence experiments for CTX and MMC are presented in Fig. 6A,B. For both drugs the largest TGT was observed when drug was given 15 min prior to heat. With this combination the observed TGT was larger than the sum of the TGTs of the two treatments given separately.

In Fig. 7A,B, dose–effect curves for maximum tolerated doses of CTX and MMC given 15 min prior to heat treatment at 43.5°C are presented in comparison with the corresponding curve for heat alone. The corresponding slopes and DERs are given in Table 3. The DERs for both CTX and MMC with either effect parameter are significantly larger than 1.0. For CTX the DER achieved with SGD as the effect parameter is substantially larger than with TGT.

In Fig. 8A,B, dose–effect curves for CTX and MMC alone and for either drug given 15 min prior to 30 min heat treatment at 43.5°C are presented. The corresponding slopes and TERs for either drug are presented in Table 4. For both drugs, with either effect parameter, the TERs are significantly larger than 1.0. For CTX the TER achieved with SGD as effect parameter, is, analogous to the DER data, substantially larger than with TGT. For MMC there are only small differences between the two effect parameters with respect to both DER and TER.

DISCUSSION

The choice of 43.5°C as treatment temperature was because the experiments were designed as a study of *local* as opposed to whole body thermochemotherapy [1–5]. In agreement with previous data with this animal tumour model [7], a linear dose–effect relationship was observed between heat

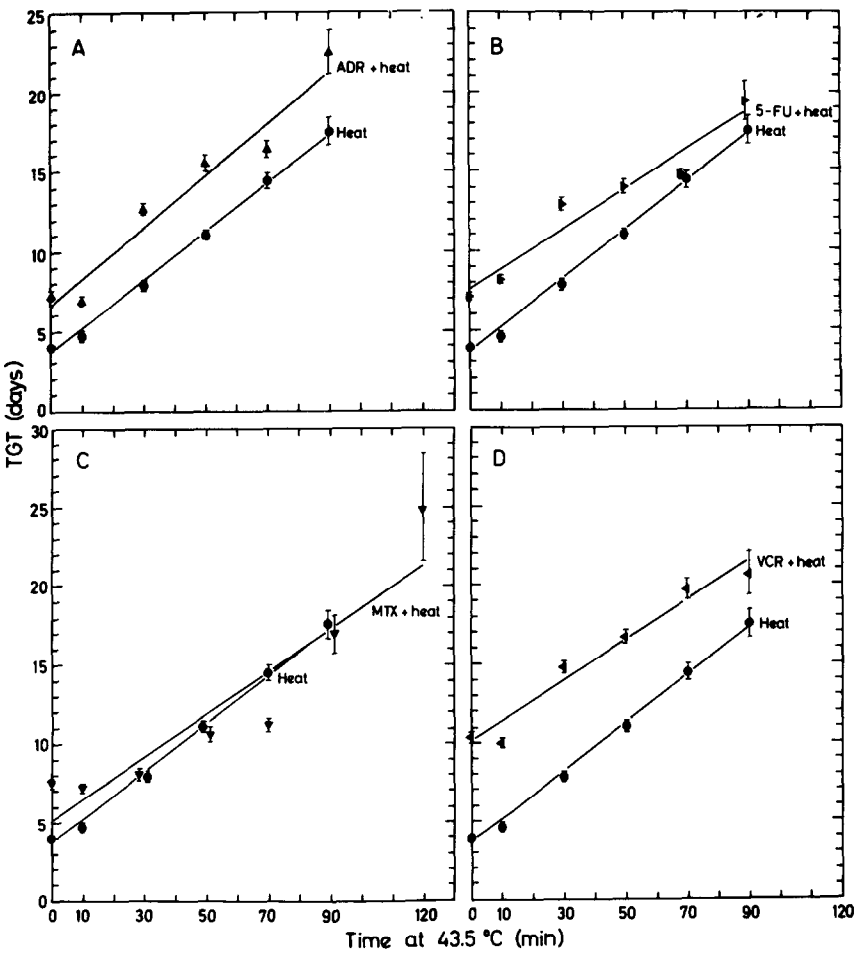

Fig. 5. Dose–effect curves using TGT as effect parameter for heat treatment alone at 43.5°C and MTD of ADR, 5FU, MTX and VCR given 15 min prior to heat treatment at 43.5°C. The points and bars are the mean TGTs and standard errors of the mean. Linear regression analysis was based on individual TGTs of all contributing tumours.

Table 2. Drug enhancement ratios (DER) after single fraction thermochemotherapy with maximally tolerated drug doses using two different effect parameters

Treatment	Tumour growth time (TGT)			Specific growth delay (SGD)		
	Slope ± S.D.	DER ± S.E.	Level of significance	Slope ± S.D.	DER ± S.E.	Level of significance
Heat alone	0.152 ± 0.005			0.071 ± 0.002		
ADR + heat	0.165 ± 0.011	1.1 ± 0.1	n.s.	0.074 ± 0.006	1.0 ± 0.1	n.s.
5FU + heat	0.125 ± 0.008	0.8 ± 0.1	n.s.	0.061 ± 0.006	0.9 ± 0.1	n.s.
MTX + heat	0.135 ± 0.013	0.9 ± 0.1	n.s.	0.055 ± 0.006	0.8 ± 0.1	$P < 0.05$
VCR + heat	0.127 ± 0.009	0.8 ± 0.1	n.s.	0.054 ± 0.006	0.8 ± 0.1	$P < 0.05$

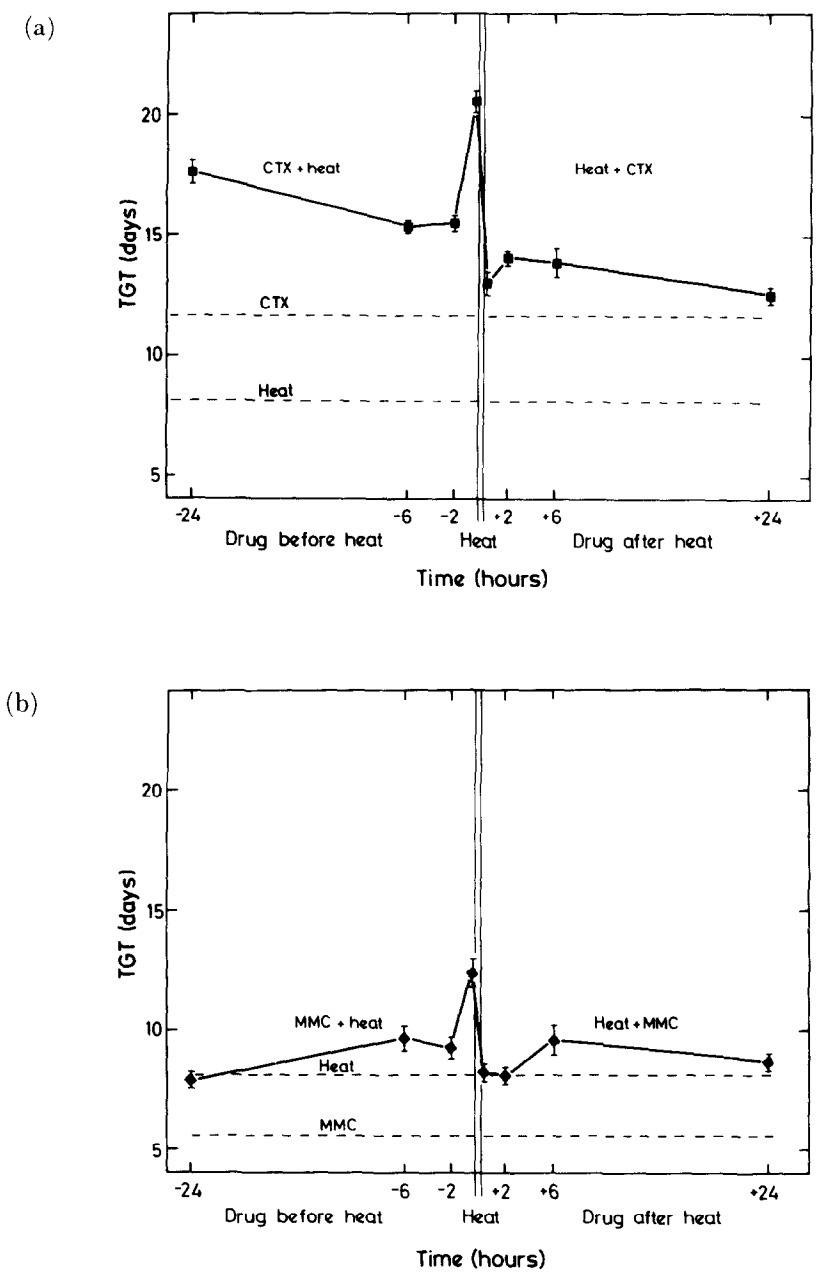

Fig. 6. Effect of timing and sequence of thermochemotherapy using TGT as effect parameter. Maximum tolerated drug doses are given either 24, 6, 2 h *or* 15 *min before or after* 30 *min heat treatment at* 43.5°*c. a: ctx* 100 mg/kg. *B: MMC* 3 mg/kg. *Broken lines are the effects of either heat alone* (43.5°C, 30 *min*) *or MTD of each drug. The points and bars are the mean TGTs and standard errors of the mean.*

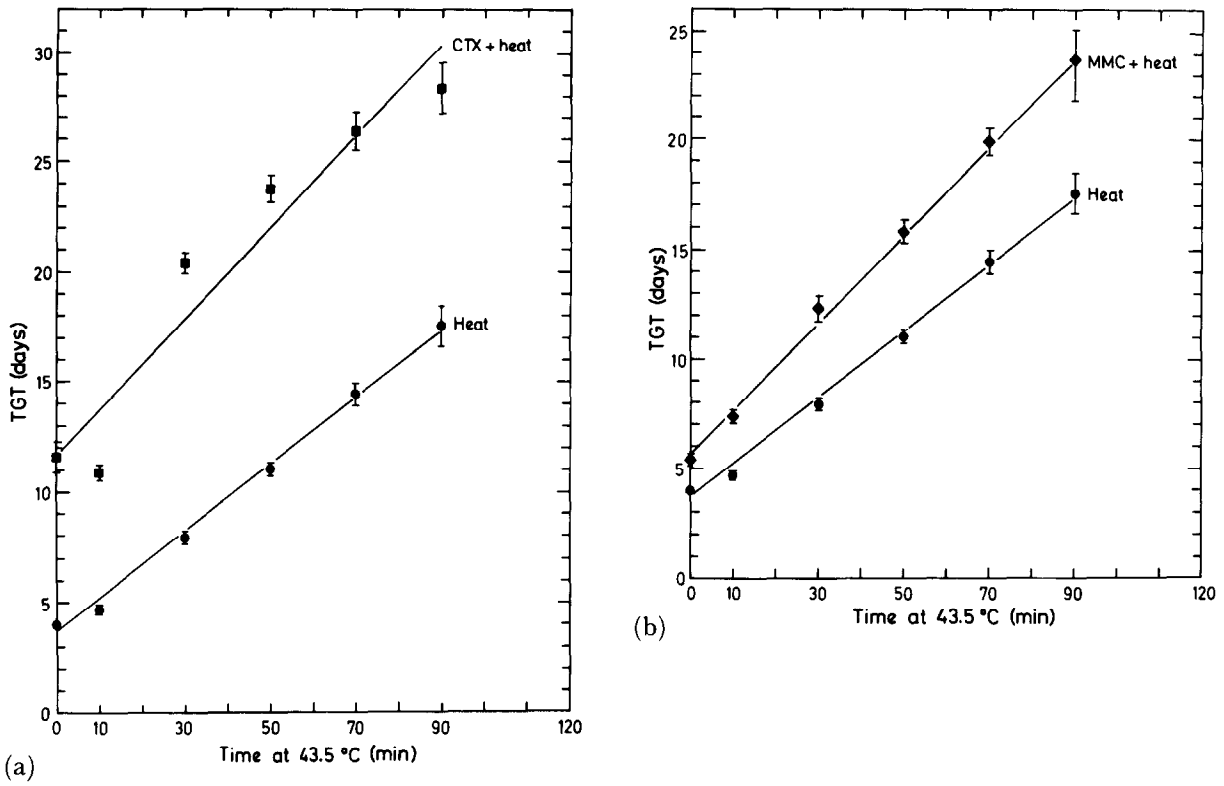

Fig. 7. Dose–effect curves using TGT as effect parameter for heat treatment alone at 43.5°C and MTD of CTX and MMC given 15 min prior to heat treatment at 43.5°C. The points and bars are the mean TGTs and standard errors of the mean. Linear regression analysis was based on individual TGTs of all contributing tumours. A: CTX. B: MMC.

Table 3. Drug enhancement ratios (DER) after single fraction thermochemotherapy with maximally tolerated drug doses using two different effect parameters

	Tumour growth time (TGT)			Specific growth delay (SGD)		
Treatment	Slope ± S.D.	DER ± S.E.	Level of significance	Slope ± S.D.	DER ± S.E.	Level of significance
Heat alone	0.152 ± 0.005			0.071 ± 0.002		
CTX + heat	0.207 ± 0.011	1.4 ± 0.1	$P << 0.001$	0.152 ± 0.006	2.1 ± 0.1	$P << 0.001$
MMC + heat	0.201 ± 0.011	1.3 ± 0.1	$P < 0.01$	0.097 ± 0.003	1.4 ± 0.1	$P << 0.01$

dose and the chosen effect parameters within the dose range studied. The thermochemotherapy and drug alone data could also be fitted to linear regression lines in dose–effect plots, allowing enhancement ratios to be expressed as ratios of slopes of dose–effect curves.

The tumour growth rate was faster in the present experiments than in the laboratory from which the tumour was obtained [7, 10]—the tumour volume doubling time of untreated controls was approx. 1.8 days compared to 2.4 days in Overgaard's laboratory. However, a number of seemingly minor differences in experimental conditions may be of importance for tumour growth characteristics and may have contributed to the observed differences in growth rate, e.g. dietary composition, environmental temperaure, number of tumour passages, tumour volume and host sex [11–14].

The thermometry experiments confirmed, with two types of probes (Fig. 1), previous data from this animal tumour system that tumour core temperature stabilizes approx. 0.2°C below the water bath temperature [6]. It is known from other studies [15–18] that thermal gradients within water-heated experimental tumours depend on several factors, e.g. tumour type, size and site, water bath temperature, ambient temperature, regulation of animal core temperature and the type of anaesthesia employed. The mentioned experimental conditions were well controlled throughout the present study.

The drug doses employed [8, 19] were well tolerated and resulted in only minor and transient weight losses. Thus, tumour growth retardation due to animal weight loss [20] is not likely to explain

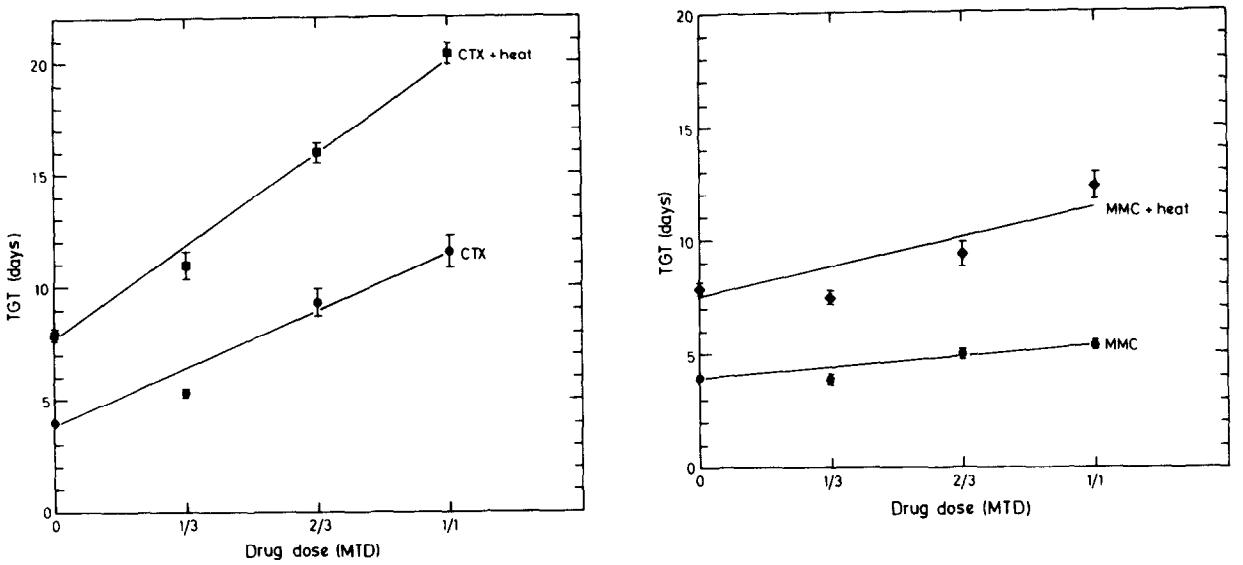

Fig. 8. Dose–effect curves using TGT as effect parameter for drug alone or drug given 15 min prior to 30 min heat treatment at 43.5°C. The points and bars are the mean TGTs and standard errors of the mean. Linear regression analysis was based on individual TGTs of all contributing tumours. A: CTX, doses up to 100 mg/kg. B: MMC, doses up to 3 mg/kg.

Table 4. Thermal enhancement ratios (TER) after single fraction thermochemotherapy using two different effect parameters

	Tumour growth time (TGT)			Specific growth delay (SGD)		
	Slope ± S.D.	TER ± S.E.	Level of significance	Slope ± S.D.	TER ± S.E.	Level of significance
CTX + 43.5°C 30 min	12.365 ± 0.491			6.543 ± 0.309		
		1.6 ± 0.1	$P << 0.001$		2.7 ± 0.2	$P << 0.001$
CTX	7.576 ± 0.325			2.463 ± 0.088		
MMC + 43.5°C 30 min	3.830 ± 0.510			1.751 ± 0.288		
		2.8 ± 0.5	$P << 0.001$		3.3 ± 0.7	$P << 0.001$
MMC	1.377 ± 0.155			0.531 ± 0.059		

the treatment effects observed in this study. The observed differences in DT between treatment groups were, however, drug-induced and the consequences of the observed variations in DTs for the course of dose–effect curves are accounted for by the introduction of SGD as a second effect parameter [14, 21, 22].

For both CTX and MMC the timing and sequence data in Fig. 6 indicate that drug given 15 min prior to heating resulted in the largest tumour response. This response is larger than the sum of the two treatments given separately. This finding is comparable to data from other *in vivo* thermochemotherapy studies with CTX [23] and other drugs [4, 5]. The present study is the first report of the effect of timing and sequence on the interaction between heat and MMC in a tumour. Other studies indicate that it is the presence of drug within cells during heating that is responsible for the enhanced effects observed from combined treatment [1–5]. The observation of maximum tumour effect with drug given shortly prior to heat is probably—due to pharmacokinetic phenomena—an effect of a simultaneous interaction of heat and drug at the cellular level during heating [1–5]. In the dose–effect experiments all drugs were given 15 min prior to heating.

There was conformity between TGT–DERs for ADR, 5FU, MTX and VCR—none being significantly different from 1.0. The dissimilarities between TGT–DERs and SGD–DERs for the four drugs were generally small, but the SGD–DERs for MTX and VCR indicate the possibility of negative drug enhancement of the tumour response to heat. However, the finding of a discrepancy between the two sets of DERs for the two drugs calls for a cautious interpretation. Our interpretation of the present body of data for the four drugs is that there

has been no measurable drug enhancement of the tumour response to heat treatment at 43.5°C of any of the four drugs [24–26].

For CTX and MMC the finding that all enhancement ratios were significantly larger than 1.0, irrespective of the effect assay employed, supports the interpretation that a significant drug enhancement of the tumour response to heat, as well as a significant heat enhancement of the tumour response to drug, has taken place for both drugs [24–26]. Particularly for CTX there was variation between TGT-derived and SGD-derived enhancement ratios. This reflects the observation of larger variation in DTs among CTX-treated tumours than among MMC-treated. For CTX, the SGD-derived enhancement ratios were larger than the corresponding TGT-derived ratios. For this drug, TER was larger than DER, but the differences were comparatively small when ratios estimated with the same effect parameter are compared. A reasonable interpretation of the CTX data is that both DER and TER are in the range of approx. 1.5–2.5. For MMC, the thermal enhancement of the drug effect seems to be considerably larger than the drug enhancement of the heat effect; DER is approx. 1.3–1.4 and TER approx. 3.0.

In the literature there have been reports of both enhanced and a lack of enhanced responses of combined treatments *in vivo* [1–4]. A variety of effect parameters are used, but there have been comparatively few estimations of enhancement ratios based on comparison between dose–effect curves [1–5, 27, 28]. Such studies are probably needed in order to substantiate the scientific basis for clinical thermochemotherapy.

There have been no previous reports of estimations of DER *in vivo* for ADR, 5FU, MTX or VCR [4, 5]. A complex interaction between ADR and heat has been described [2, 4, 5]. Overgaard found in the same animal tumour system using about 3× the drug dose as in the present experiments an increased frequency of tumour cure with combined ADR and hyperthermia of 40.5°C and 42.5°C [29]. The heat treatment seemed to protect the animals from the toxicity of the high dose of ADR. In other studies with ADR, diverging results with either an enhanced or lack of enhanced tumour response with thermochemotherapy have been found [4, 30–32]. A mixed response pattern for thermochemotherapy *in vivo* is reported also for the antimetabolites 5FU and MTX [4, 33–36], but there are no data on thermochemotherapy with high dose MTX and folinic acid rescue. For vinca-alkaloids, there are no previous reports of enhanced tumour effects with thermochemotherapy [4, 5]. With 5FU, MTX and VCR there are no previous tumour data with temperatures above 43°C [4, 5].

Several authors have reported increased tumour and normal tissue responses with thermochemotherapy involving CTX or MMC, compared to the effects of either drug alone or heat alone [4, 5]. However, no previous estimations of DER in tumours have been found for any of the two drugs. For CTX a TER of the range 1.4–1.9 has been reported in RIF tumours in mice given whole body hyperthermia at 41.5°C [28]. In a Lewis lung carcinoma, 42.5°C for 30 min gave a TER of 1.5–1.7 depending on the assay employed [27]. For MMC no previous reports of TER in tumours have been found.

The present study with this animal tumour model for local thermochemotherapy indicates that there is, for both CTX and MMC, a significant drug enhancement of the tumour response to heat treatment and a significant thermal enhancement of the tumour response to drug treatment. The data reported support the choice of both CTX and MMC in preference to ADR, 5FU, MTX or VCR for clinical thermochemotherapy studies.

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