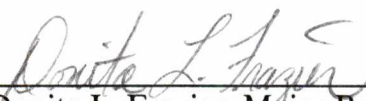


To the Graduate Council:

I am submitting herewith a dissertation written by August V. Wilke, III entitled "The Effects of Hyperthermia and Heat-Shock Protein Expression on Adriamycin Pharmacokinetics." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

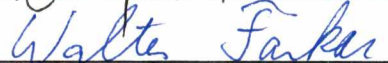


Donita L. Frazier, Major Professor

We have read this dissertation
and recommend its acceptance:







Accepted for the Council:



Associate Vice Chancellor
and Dean of The Graduate School

The Effects of Hyperthermia
and Heat-shock Protein Expression on Adriamycin Pharmacokinetics

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

August V. Wilke, III

August 1991

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This dissertation is dedicated to my wife, Teresa, and to our families.

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ABSTRACT

The effect of hyperthermia on the pharmacokinetics of the antineoplastic drug, Adriamycin, was examined. The distribution of Adriamycin to the tissues was increased in dogs treated with mild whole-body hyperthermia (42°C for 1 hr). Although peak duodenal drug concentration was increased in dogs treated with concurrent hyperthermia, no overt toxicity to the duodenum was demonstrated using select enzyme assays or histologic examination. A slight, nonstatistically significant depression of cell turnover was demonstrated in duodenal biopsies from dogs treated with Adriamycin or Adriamycin combined with whole-body hyperthermia.

The effect of local hyperthermia (43.5°C for 1 hr) on Adriamycin concentration in an *in vivo* rat mammary tumor model was also examined. Prior hyperthermia was seen to reduce Adriamycin concentrations in tumors when Adriamycin was administered 3 hr after local hyperthermia. The production of HSP 70 was also demonstrated in tumors 3 hr after 1 or 2 hyperthermia treatments. Decreased Adriamycin concentration was not due to decreased blood flow to tumors following local hyperthermia as demonstrated by radioactive microsphere techniques.

In conjunction with the above studies an HPLC method based on fluorescence detection was developed for small tissue biopsies, plasma, and tumor samples. A fluorescence-activated cell sorter (FACS) technique was also developed for Adriamycin determination in tumor samples. A new method for use of a Nd:YAG laser with accompanying surface cooling to heat superficial tumors is also described.

TABLE OF CONTENTS

CHAPTER	PAGE
Part 1: LITERATURE REVIEW AND GENERAL INTRODUCTION	
1. HYPERTHERMIA.....	2
History/General Review of Techniques.....	2
Hyperthermic Cytotoxicity/Rationale for Hyperthermia.....	4
Interaction of Hyperthermia and Adriamycin.....	5
2. THERMOTOLERANCE.....	9
Description of Thermotolerance.....	9
Genetic Regulation of HSP Expression.....	11
Transcriptional Control in Prokaryotes.....	11
Transcriptional Control in Eukaryotes.....	14
Posttranscriptional Control in Eukaryotes.....	15
Translational Control in Eukaryotes.....	16
3. ADRIAMYCIN.....	17
Source/Structure.....	17
Use in Cancer Chemotherapy.....	17
Limitations to Use.....	20
Mechanisms of Action.....	22
Distribution Kinetics.....	24
Metabolism.....	24
Elimination.....	25
Literature Cited.....	27
Appendix.....	32
Part 2: THE EFFECT OF HYPERTHERMIA ON NORMAL TISSUE TOXICITY AND ON ADRIAMYCIN PHARMACOKINETICS IN DOGS	
1. INTRODUCTION.....	36
2. MATERIALS AND METHODS.....	37
Adriamycin Pharmacokinetics.....	37
Cell Turnover.....	39
Histopathology.....	39
Enzyme Assays.....	40
3. RESULTS.....	41
Pharmacokinetics.....	41
Cell Turnover.....	42
Histopathology and Clinical Parameters.....	42
Enzyme Assays.....	43
4. DISCUSSION.....	44
5. LITERATURE CITED.....	48
6. APPENDIX.....	50

Part 3: THE EFFECT OF HSP 70 EXPRESSION ON ADRIAMYCIN
PHARMACOKINETICS IN AN *IN VIVO* RAT MAMMARY
TUMOR MODEL

1.	INTRODUCTION.....	58
2.	MATERIALS AND METHODS.....	59
	Tumor Model.....	59
	Hyperthermia.....	59
	HSP 70 Expression.....	60
	Blood Flow	60
	Adriamycin	61
3.	RESULTS.....	63
	HSP 70.....	63
	Blood Flow	63
	Adriamycin Determinations Using HPLC	64
	Adriamycin Determinations Using FACS.....	65
4.	DISCUSSION	66
5.	LITERATURE CITED	68
6.	APPENDIX.....	70

Part 4: RELATED STUDIES

1.	DETERMINATION OF ADRIAMYCIN IN TISSUES AND PLASMA	76
2.	ND:YAG LASER HYPERTHERMIA OF A RAT MAMMARY ADENOCARCINOMA WITH ACCOMPANYING SURFACE COOLING.....	92

Part 5: SUMMARY

1.	CONCLUSIONS	100
2.	FUTURE DIRECTIONS.....	102
	VITA	103

LIST OF TABLES

TABLE		PAGE
Part 2:	THE EFFECT OF HYPERTHERMIA ON NORMAL TISSUE TOXICITY AND ON ADRIAMYCIN PHARMACOKINETICS IN DOGS	
1.	Pharmacokinetic parameters describing the disposition of adriamycin in normal dogs with and without concurrent whole body hyperthermia (42°C).....	51
2.	Enzyme activities of duodenal biopsies from normal dogs receiving adriamycin, 42°C whole body hyperthermia for 1 hour or both concurrently.....	52
Part 4:	RELATED STUDIES	
1.	Within assay precision for spiked tissue standards. Spiked concentration (Concentration, ng Adriamycin/5 mg tissue) and the mean of experimentally determined concentrations (Mean) are shown.....	85
2.	Between-day assay precision for spiked tissue standards. Spiked concentration (Concentration, ng Adriamycin/5 mg tissue), experimentally determined concentration (Measured Concentration), and the mean ratio of Adriamycin to daunomycin (Mean Area Ratio) are shown	86
3.	Between-day assay precision for spiked plasma standards. Spiked concentration (Concentration, ng Adriamycin/ml plasma), experimentally determined concentration (Measured Concentration), and the mean ratio of Adriamycin to daunomycin (Mean Area Ratio) are shown	87
4.	Mean tumor temperature (°C) over 1 hour obtained using Nd:YAG laser hyperthermia with concurrent surface cooling.....	94

LIST OF FIGURES

FIGURES	PAGE
Part 1: LITERATURE REVIEW AND GENERAL INTRODUCTION	
1. Metabolism of Adriamycin to its major metabolites.....	33
2. Adriamycin redox cycling.....	34
Part 2: THE EFFECT OF HYPERTHERMIA ON NORMAL TISSUE TOXICITY AND ON ADRIAMYCIN PHARMACOKINETICS IN DOGS	
1. Representative plots of actual ADR and ADR-OL plasma concentrations in dogs receiving drug alone and drug with concurrent whole body hyperthermia	53
2. Plasma adriamycin concentration versus time curves generated from pharmacokinetic data using the formula: $C_{ADR} = A \cdot e^{-(a \cdot t)} + B \cdot e^{-(b \cdot t)}$	54
3. Adriamycin content of duodenal biopsies (mean $\pm$ SEM; n=5). Biopsies were collected immediately after and one hour after drug administration from dogs receiving drug alone (ADR) or drug concurrent with 42°C hyperthermia (ADR + WBH).....	55
4. ³ H-Thymidine uptake by duodenal biopsies incubated for one hour in the presence of 0.5 μ Ci ³ H-thymidine. (mean $\pm$ SEM; n=5).....	56
Part 3: THE EFFECT OF HSP 70 EXPRESSION ON ADRIAMYCIN PHARMACOKINETICS IN AN <i>IN VIVO</i> RAT MAMMARY TUMOR MODEL	
1. ³ H-methionine incorporation in HSP 70 from tumors heated 1 or 2 times (43.5°C for 1 hour)	71
2. Blood flow rates to rat mammary tumors as determined by ¹¹³ Sn microsphere injection.....	72
3. Adriamycin concentrations in rat mammary tumors 30 or 60 minutes after injection as determined by HPLC.....	73
4. Adriamycin concentrations in rat mammary tumors 30 or 60 minutes after injection as determined by FACS.....	74

Part 4: RELATED STUDIES

1. Chromatogram of a mixture of adriamycinol (2), adriamycin (3), and daunomycin (4) in tissue (100 ng/5 mg spiked tissue)..... 88
2. Chromatogram of a tissue sample collected 60 minutes after administration of 30 mg/m² adriamycin to a canine. Endogenous tissue components (1), adriamycin (2), and daunomycin (3) were detected..... 89
3. Chromatogram of a mixture of adriamycinol (2), adriamycinol aglycone (3), adriamycin (4), adriamycin aglycone (5), and daunomycin (6) (100 ng/ml spiked plasma). Peak one results from endogenous plasma components 90
4. Chromatogram of a plasma sample collected 15 minutes after administration of 30 mg/m² adriamycin to a canine. Endogenous plasma components (1), adriamycinol (2), adriamycin (3), and daunomycin (4) were detected..... 91
5. The effect of laser hyperthermia with and without surface cooling on temperature just under the skin in a rat mammary adenocarcinoma 96
6. The effect of laser hyperthermia with and without surface cooling on temperature in the center of a rat mammary adenocarcinoma 97
7. The effect of laser hyperthermia with and without surface cooling on temperature at the base of a rat mammary adenocarcinoma 98

PART 1

LITERATURE REVIEW AND GENERAL INTRODUCTION

CHAPTER 1: HYPERTHERMIA

History/General Review of Techniques

The first indication of the efficacy of hyperthermia against cancer is contained in a report by Busch in 1866. This report details the disappearance of a spontaneous tumor after a natural *Streptomyces* infection-induced fever (Hornback, 1989). Several similar cases were later documented by others. The next major step, although it was not appreciated as such, was a report in 1893 by Coley on bacterial toxin therapy. Coley induced fever ($38-40^{\circ}\text{C}$ for 24-36 months) in cancer patients by injecting them with bacterial toxin. Retrospective studies on his inoperable cancer cases have shown a five year survival rate of almost 60% (Hornback, 1989). In retrospective studies Miller and Nicholson (1971) reported that, in order to see a response, temperatures in Coley's patients needed to be at least 38.3°C for 6 to 12 months. Coley, however, probably missed the significance of these results since his rationale for the therapy was that bacterial toxin acted directly on the macroparasitic organisms he thought caused cancer.

In modern times interest in hyperthermia was initiated by Pettigrew (1971) who developed a molten wax bath for heating patients. Since then hyperthermia methods utilizing radiant heat devices, heated water blankets, arteriovenous shunts, radiofrequency, microwaves, ultrasound and contact lasers have been developed. A brief overview of the characteristics of these as well as their application in local, regional or whole-body hyperthermia (WBH) follows.

WBH has been induced using the least technologically sophisticated methods, radiant heat and water blankets. In these methods the patient is

placed inside the unit or beneath a heated water blanket, then the temperature is gradually raised. Approximately 90 minutes is required to heat a human to 42°C (Grisham and Barnett, 1973). These methods have significant, although reversible, side effects, including parathesia, alterations in clotting parameters, and alterations in liver enzyme activities (Dewhirst, 1987). Prohibitive systemic toxicities have been reported in human patients with advanced malignancies (Dewhirst, 1987). A relatively large number of human patients have been heated using arteriovenous shunt techniques. Side effects associated with this method include cerebral and pulmonary edema as well as muscular weakness after treatment. (Parks, 1982). Cerebral edema and weakness were found to be alleviated with parenteral K₂PO₄ administration, while intravenous crystalloids and positive end expiratory pressure prevented pulmonary edema. Systemic toxicities limit the temperature used in any WBH, even in normal individuals. Life threatening complications including hemostatic aberrations, liver failure, cardiac decompensation, and seizures have been reported in dogs heated to above 43°C (Rosenthal et al., 1971; Shapiro et al., 1973). Pettigrew et al. (1974) report a death due to ventricular fibrillation after core temperature in a human patient exceeded 43°C. Thrall et al. (1990) reported that WBH resulted in lower, but more uniform temperatures compared with local hyperthermia in solid canine tumors. That study obtained the higher and more uniform temperatures using a combination of WBH (radiant) and local, radiofrequency hyperthermia.

Hyperthermic Cytotoxicity/Rationale for Hyperthermia

It has been suggested that the effects of hyperthermia can be classified as either direct cytotoxicity or sensitization to radiotherapy and/or chemotherapy (Oleson and Dewhirst, 1983). Direct cytotoxicity may be the result of increased fluidity of the plasma membrane. Changes in the conformation of plasma membrane proteins during the transition from 38-45°C have been documented using Raman spectroscopy (Wallach et al., 1979). The proposed role of the plasma membrane is supported by the finding that membrane active drugs, such as aliphatic alcohols (Hahn and Li, 1982) and local anesthetics (Juvekar et al., 1987), potentiate hyperthermia. Several multi-molecular complexes, including the insulin receptor (Calderwood and Hahn, 1983), and the mitochondrial electron transport chain (Bowler, 1981), have been shown to be sensitive to heat. Transcription initiation (Parker and Topol, 1984), RNA splicing (Toot and Lindquist, 1986), and translation initiation (Didomenico et al., 1982) are also interrupted by heat and may play a role in direct cytotoxicity. One study has reported that the cytoplasmic matrix and, in particular, the intermediate filaments are susceptible to heat, collapsing into a perinuclear zone after hyperthermia (Welch et al., 1985).

The rationale for the greater heat sensitivity of tumor cells relative to normal cells lies primarily in the nature of tumor vasculature. Tumors have a fixed arteriolar blood supply and, therefore, recruit venous and capillary vessels. These recruited vessels are often relatively fragile and often have incomplete endothelial linings (Peterson, 1979). This results in a hypotensive, sluggish, often regurgitant blood supply and causes tumor cells to be hypoxic and acidotic

due to reduced movement of nutrients and metabolic wastes. Low pH has been shown to inhibit the development of thermotolerance in certain cell lines (Gerner, 1983). It has been observed that 1) hyperthermia preferentially kills hypoxic/acidotic cells, 2) hyperthermia preferentially destroys tumor microvasculature, resulting in further reduction in blood supply, and 3) tumors heat more easily than normal tissue due to their sluggish blood flow.

Interaction of Hyperthermia and Adriamycin

The first report of an *in vitro* interaction of heat with ADR is provided by Hahn et al. (1975) who examined survival of EMT-6 cells after simultaneous exposure to ADR and heat. Although 37°C, 41°C, and 42°C were used, only 42°C caused significant reduction in survival. An additional study (Hahn and Strande, 1976) demonstrated that this phenomenon had time dependency: simultaneous heating in the range of 30-60 minutes produced maximum synergy with ADR, while longer heating caused resistance to ADR. This study also demonstrated that ADR resistance was seen in cells heated to 42°C, then exposed to ADR at 37°C.

ADR resistance after heat was confirmed by other groups including Morgan et al. (1979) who observed resistance to ADR at 37°C after 43°C, but not after 40°C for 3 hours. Using the FM3A cell line, Mizuna et al. (1980) documented ADR sensitivity only during the first 30 minutes at 43°C followed

by ADR resistance. Dahl (1982) reported a 1 hour 41°C hyperthermia-induced ADR sensitivity, followed by ADR resistance if heating continued in BT4-C. An additional feature has been reported by Herman et al. (1982) who demonstrated that ADR/hyperthermia synergy decreased with slower heating.

Several *in vitro* studies present evidence that this synergism is due to increased influx of ADR into heated cells (Engelhardt, 1987). Luk and Tannock (1989) found a strong correlation between intracellular ADR concentration and cytotoxicity across seven cell lines of rodent and human origin. Rice and Hahn (1987) also were able to correlate increased cytotoxicity in CHO cells with increased intracellular ADR concentrations using FACS. Rice and Hahn found that, while simultaneous 45.5°C heat and ADR exposure led to increased intracellular ADR concentrations and increased cytotoxicity, heat followed by ADR exposure led to decreased ADR uptake and cytotoxicity for up to 12 hours. Additionally, Rice and Hahn demonstrated that increased intracellular ADR concentrations are the result of increased influx of drug, not decreased efflux. Increased intracellular concentrations of ADR with simultaneous heat *in vitro* are also reported by Mizuno and Ishida (1982), Yamanee et al. (1984), and Klein et al. (1977). Conflicting results are reported based on comparisons of ADR-sensitive and ADR-resistant sublines. Van der Linden et al. (1984) report increased ADR uptake in both ADR-sensitive and -resistant lines of P388 mouse leukemia cells, but increased cytotoxicity in the sensitive line only. Bates and Mackillop (1986) compared Chinese hamster ovary cell lines which were ADR-

sensitive (Aux B1) and -resistant (CH^R C5, which overexpresses p-glycoprotein). In Aux B1, they reported that simultaneous heat in the range of 37-45°C dramatically increased ADR uptake and only moderately increased efflux which led to increased intracellular concentration and cytotoxicity. In CH^R C5, heat did not increase ADR influx or cytotoxicity, although this might be difficult to discern if p-glycoprotein pumps ADR from cells at an accelerated rate.

ADR is thought to enter cells via passive diffusion and then be pumped out in an energy-dependent manner (Inaba et al., 1979), possibly by p-glycoprotein, the overexpression of which can lead to multi-drug resistance (MDR) (Bradley et al., 1988). In light of this, as well as the above observations, Rice and Hahn have hypothesized that heat initially increases cells' permeability to ADR, but then by some unknown mechanism, permeability is decreased. The finding by Li and Hahn (1978) that cells made thermotolerant not only by heat, but also by ethanol are ADR resistant indicates a possible involvement of heat-shock proteins.

In vivo animal studies are less numerous and less promising, often failing to demonstrate synergism or doing so only at an excessive dose. Hahn et al. (1975) reported enhanced cytotoxicity of hyperthermia (43°C for 30 minutes) with simultaneous ADR (10-25 mg/kg) against EMT-6 tumors that were transplanted into mice. Overgaard (1976) reported increased cure rates of mice with transplanted tumors after 42.5°C for 1 hour with ADR (25 mg/kg). The fact that this dose of ADR killed 90% of the control animals makes the results

seem less relevant. Marmor et al. (1979) also studied mice transplanted with EMT-6 tumors, reporting enhancement of ADR by 43°C, 30 minute hyperthermia. This effect was seen only when ADR was administered at a rate of 10 mg/kg, not 2.5 mg/kg or 5 mg/kg. Unfortunately, this combination caused 14 of 25 animals to die within 4-7 days.

CHAPTER 2: THERMOTOLERANCE

Description of Thermotolerance

Thermotolerance (TT), as defined by Hahn and Li (1990), is a "transient, acquired heat resistance." It was first recognized as an issue in cancer therapy by Gerner and Schneider (1975) and Henle and Leeper (1976) who produced this condition in CHO cells. It was observed that if cells were heated (45°C for 15 min), then returned to 37°C for about 8 hours, a greater proportion would survive subsequent 45°C heating administered up to at least 48 hours later.

It was also observed that synthesis of DNA, RNA, and protein stops during 45°C hyperthermia, then, at normal resting temperature, protein synthesis resumes with kinetics similar to TT development. In contrast, protein synthesis continues during hyperthermia below 43°C and TT can develop during hyperthermia. The "heat-shock proteins" (HSP's) produced were isolated and attempts were made to positively link their production to TT (Henle and Dethlefsen, 1978).

Several *in vitro* studies (Hahn and Li, 1982; Tomasovic, et al., 1983; Ketis, et al., 1988; Li and Mak, 1989) and at least one *in vivo* study (Li and Mak, 1985) have linked synthesis of a 70 kDa heat-shock protein (HSP 70) with TT. A notable exception is the study of Anderson, et al. (1988), where slow heating of Chinese hamster ovary cells (37-42°C over 3 hours) produced TT that did not necessarily correlate with HSP 70.

These studies have also led to a hypothetical model of TT (Hahn and Li, 1990). Based on evidence described above, Hahn and Li suggested that development of TT first involves cells being activated by heat or certain

chemicals (see below). A second step, which probably involves HSP production, and which can occur only below 43°C in rodent cells or below 44°C in human cells, must occur before TT is manifested. The decay of TT is also temperature sensitive having kinetics that suggest enzymatic degradation (Li and Hahn, 1980). This model explains the successful use of "step-down heating" where cells are briefly heated to 45°C, which blocks protein synthesis for at least 2 hours (Hahn and Shiu, 1985). Cells are then returned to 43°C (rodent cells) to which they are sensitized by their inability to synthesize proteins and develop TT (Hahn and Li, 1990).

As mentioned above, certain chemicals can induce TT. These include sodium arsenite, thiols such as carbonyl cyanide *m*-chlorophenyl hydrazone, and diamide (Henle, 1987) as well as ethanol (LI, 1988; Li and Hahn, 1978) and other alcohols (Li and Hahn, 1990). These agents mimic heat in both the kinetics and temperature permissiveness of TT development (Hahn and Li, 1990). It has also been documented that ethanol-induced TT leads to Adriamycin resistance just as heat-induced TT does. (Li and Hahn, 1978). Transient ischemia has been observed to induce HSP 70 (Nowak, 1985) and TT has been shown to speed postischemic ventricular recovery in rat hearts (Currie et al., 1988). HSP's are also induced in cells subjected to glucose deprivation or hypoxia, (Hahn and Li, 1990) and in freshly sliced tissues (Currie and White, 1981). X-irradiation does not induce the heat shock response (Anderson et al., 1988).

Genetic Regulation of HSP Expression

Transcriptional Control in Prokaryotes

Two distinctly different strategies are seen regarding the regulation of HSP production in prokaryotes as compared to eukaryotes. In order for the genetic code contained in prokaryotic DNA to be turned into protein, it must first be transcribed into mRNA. Control of this step is the primary control point in prokaryotic systems. Transcription is accomplished by RNA polymerase holoenzyme, which is composed of two parts, core RNA polymerase (designated E) and a σ factor. The σ factor causes the complex to bind to the promoter of a certain gene or family of genes and, therefore, transcribe those genes.

Eukaryotic systems are more complex, potentially exhibiting control at several levels. In the case of all HSP genes, a short segment known as the heat shock element (HSE) precedes the 5' end of the gene. For transcription to take place, the HSE must be recognized by a low molecular weight protein known as the heat shock transcription factor (HSF). Binding of HSF to HSE allows transcription to take place; in its absence, HSP production does not occur. Most eukaryotic RNA is first produced containing noncoding regions known as introns. These must be excised and the coding regions spliced together before translation into protein can take place.

The production of HSP's in *E. coli* has been traced back to the gene *rpoH*. The significance of this gene was realized in studies of *rpoH* mutants which are unable to mount a heat shock response (Gross et al., 1990). *rpoH* has four known promoters: P1, P3, P4, and P5. P1 and P4 are recognized by $E\sigma^{70}$ and are thought to be responsible for most transcription under normal conditions; P4 transcription is somewhat heat-inducible. P5 is also recognized by

$E\sigma^{70}$, but is active only when the media lacks glucose or when ethanol is present. P3 differs from the other promoters as it is inducible only by temperatures at which $E\sigma^{70}$ is thought to be inactive (50°C). This observation led to the discovery of a 24 kDa σ factor designated σ^{24} (Erickson and Gross, 1989). It is thought that σ^{24} may control a previously unknown set of HSP's (Gross et al., 1990)

σ^{32} is the product of *rpoH* which was discovered after study of *rpoH* mutants that constitutively express HSP's, but have no heat-inducible response (Neidhardt and VanBogelen, 1981; Yamamori and Yura, 1982). σ^{32} directs core RNA polymerase to recognize HSP promoters, allowing preferential expression of HSP's. Strains deficient in σ^{32} produce virtually no detectable HSP's, although $E\sigma^{70}$ transcribed mRNA's are produced at normal rates (Zhou et al., 1988). Experiments in other labs have demonstrated that $E\sigma^{70}$ does not bind to promoters of the HSP genes *groE*, *dnaKP1*, *dna KP2*, or *htprG* (Cowing et al., 1985; Fujita et al., 1987). Taken together, these experiments indicate there is little, if any, overlap in σ^{32} vs. σ^{70} function.

DNase I footprinting is often used to reveal the binding specificity of protein/DNA complexes. If protein is bound to a given region of DNA, it is protected from DNase cleavage. These experiments have given almost identical results when the $E\sigma^{32}/groE$ complex is exposed to DNase I as when the non-HSP $E\sigma^{70}/lacUV5$ complex is exposed (Cowing and Gross, 1989). In both cases, -60 through +20 base pairs (bp) are protected as are -10 and -35, while the region upstream of -35 shows enhanced cleavage. This indicates that the promoters might have similar sequences and that the $E\sigma$ complexes possibly have similar binding properties. It has, however, been demonstrated that the polymerase/promoter complexes are not identical in that they have different

tolerances for dissociation. $E\sigma^{32}$ /HSP promoters can be destabilized by heparin, poly [d(AT)], and poly [(IC)] while $E\sigma^{70}$ is not, implying that they are structurally distinct (Cowing, 1988).

rpoH mRNA is abundant, but σ^{32} levels are normally low indicating that this probably represents a negatively regulated system (Straus et al., 1987). Once an appropriate temperature is reached, some signal causes repression to be lost and σ^{32} is synthesized. Additional evidence is supplied by gene fusion experiments with lacZ using β -galactosidase as a reporter. When both rpoH transcription and translation controls were fused to lacZ, β -galactosidase production after heat shock followed kinetics similar to that of σ^{32} , but when only transcription control was included, no β -galactosidase was produced (Straus et al., 1987).

E. coli experience a 10-20 fold increase in HSP production during the first 5 minutes after a temperature upshift from 30°C to 42°C (Gross et al., 1990). This phenomenon is probably due to an increase in HSP mRNA because the transcription inhibitor, rifampicin, blocks HSP production (Yamamori and Yura, 1980). This finding is supported by studies on specific HSP mRNA production rates using reporter gene splicing techniques (Yano, 1987) and by studies on accumulation of specific HSP mRNA's (Taylor et al., 1984). The most conclusive evidence is provided by immunological detection of σ^{32} levels, which demonstrate that σ^{32} is linked to increased initiation at heat shock promoters (Lesley et al., 1987; Skelley et al., 1987; Straus et al., 1987). This increase in σ^{32} accumulation is due in part to a transient increase in its stability (Gross et al., 1990). While σ^{32} is labile during steady-state growth at either 30°C or 42°C ($t_{1/2}$ =1 min.), it is stabilized during the first 4-5 minutes after temperature upshift (Straus et al., 1987).

σ^{32} synthesis and degradation somehow involves three HSP's: DnaK, DnaJ, and GrpE (Gross et al., 1990). In general, certain mutants in any of the loci coding for these proteins overexpress HSP's at 30°C and fail to shut off HSP production at 42°C (Tilly et al., 1983). After closer study, it was found that dnaK, dnaJ, and grpE mutants fail to degrade σ^{32} at 30°C and dnaK and grpE mutants fail to degrade σ^{32} after 8 minutes at 42°C (Tilly et al., 1989). This negative regulation loop is more complex than these HSP's acting as classic proteases since certain mutants, including deletion mutants, have wildtype σ^{32} levels at 30°C (Gross et al., 1990). It seems that σ^{32} degradation is heat sensitive. It is unknown whether the 3 HSP's act singly or in concert nor is it known whether they interact with a protease, σ^{32} , or a σ^{32} /protease complex (Gross et al., 1990). It has been suggested that these HSP's facilitate the dissociation of the core RNA polymerase/ σ^{32} complex making σ^{32} more susceptible to degradation (Georgopoulos et al., 1990).

Transcriptional Control in Eukaryotes

In eukaryotic systems, HSP production is first controlled by the binding of heat shock transcription factor (HSF) to heat shock elements (HSE) (Wu et al., 1990). This binding has been shown to not require protein synthesis (Zimarino and Wu, 1987). HSE is present in multiple copies and highly conserved; that is, a working copy from one organism is usually functional in another organism (Lis et al., 1990). Functional HSE was once thought to contain a 14 bp consensus sequence but is now known to consist of a 5 bp sequence: GAA or its complement TTC with each "module" inverted relative to the adjacent module

(Amin et al., 1988; Xiao and Lis, 1988). At least two modules are required with as many as 4 (HSP 70) and 8 (HSP 83) occurring in *Drosophila* (Lis et al., 1990).

HSF is thought to exist as a trimer (Sorger and Nelson, 1989), but its mechanism of transcription induction is unknown. RNA polymerase II binds at the HSP promoter and synthesizes about 25 nucleotides, but then stops until HSF presumably removes the block (Rougvie and Lis, 1988).

Posttranscriptional Control in Eukaryotes

It was observed in *D. melanogaster* that, although HSP 82 was produced after moderate heat shock, it was not produced after severe heat shock. This contrasts with other HSP's which are induced by either heat treatment. This is not due to a translation defect as shown by first applying moderate heat shock, then immediately raising the temperature into the "severe" range. HSP 82 was induced by this protocol, indicating that a lag was needed between induction of transcription and severe heat shock. The result raised the possibility that heat shock somehow blocked HSP 82 mRNA processing. Later, the HSP 82 gene was sequenced and was found to contain regions coding for introns. It had previously been established that most HSP's do not contain introns (Holmgran et al., 1979; Ingolia et al 1980).

Study of additional genes fused with the HSP 70 promoter demonstrated that a general block of mRNA splicing occurs during severe heat shock (Yost and Lindquist, 1986, 1988). This finding has since been extended to yeast (Yost and Lindquist, in prep.), chickens (Bond and Schlesinger 1986), mammals (Kay et al., 1987; Bond, 1988), and *Dictyostelium* (Maniak and Nellen, 1988). If blocked during heat shock, mRNA are not processed when cells return to

normal temperature (Yost et al., 1990). Mild pretreatment with heat will allow normal splicing to proceed during more severe heating, suggesting that one or more HSP functions in protecting the splicing mechanism (Yost and Lindquist, 1986).

Translational Control in Eukaryotes

It has long been observed that eukaryotic HSP mRNA's are preferentially translated relative to all other mRNA's during heat shock (Lindquist, 1987). Several possible explanations for this phenomenon have been tested and refuted: degradation of preexisting mRNA's, HSP mRNA competition, and heat-induced conformational changes of mRNA's (reviewed in Lindquist, 1987). Experiments using *Drosophila* HSP 70 mRNA with 5' deletions have raised the possibility that this region contains the signal allowing HSP mRNA to be translated during heat shock (McGarry and Lindquist, 1985; Hultmark et al., 1986; Lindquist, 1987). The situation is more complex, though, as deletion of the first 27 nucleotides as well as deletions in the middle of the leader do not block translation (Lindquist, 1987)

CHAPTER 3: ADRIAMYCIN

Source/Structure

Adriamycin (ADR) is an anthracycline antibiotic isolated from the mutant fungus *Streptomyces peucetius* var. *caesius* (Arcamone, 1969). It consists of a quinone-containing tetracycline ring structure attached to a daunosamine sugar group via a glycosidic linkage (Fig. 1) (Young, 1981). The tetracycline group is a conjugated system with absorbance maxima at 233, 253, 290, 477, 495, and 530 nm in methanol making solutions of ADR red-orange (Arcamone, 1969). ADR is also fluorescent, having an emission maximum at 585 nm.

ADR is soluble in water, methanol and other aqueous alcohols, but is practically insoluble in most nonpolar organic solvents such as acetone, benzene, chloroform, ethyl ether, and petroleum ether (Windholz, 1976). Solutions are stable at neutral pH, but unstable in either acidic or alkaline conditions (Arcamone, 1969). Solutions must also be stored below 5°C and protected from light. (Arcamone, 1969)

Use in cancer chemotherapy

ADR has been shown to cause responses in a broad spectrum of tumor types *in vitro*, including acute leukemia, malignant lymphomas and several solid tumors (Young, 1981). With few exceptions, however, it is not associated with cures when used as a single agent *in vivo* (Speth, 1988). It is, therefore, most often used clinically as part of a multi-drug combination regimen.

Acute leukemia is one malignancy that is responsive to ADR alone and in combination with other drugs. One study concluded that ADR is as effective as daunorubicin, a standard agent in treatment of acute leukemia (Preisler, 1977).

Hodgkin's lymphoma also responds to ADR therapy. Combination regimens are used primarily, but this disease can be cured with ADR as a single agent (Speth, 1988). Effective combinations used against Hodgkin's include "non-cross-resistant" protocols such as ABVD (ADR, bleomycin, vinblastine, dacarbazine), which gave equivalent results to those of MOPP (mechlorethamine, vincristine [Oncovin], procarbazine, prednisone) (Preisler, 1977). Similar results were obtained when radiotherapy was added to each protocol- complete responses of 80.7% and 92.4%, respectively, were obtained when radiotherapy was combined with MOPP and ABVD (Santaro, 1987). HOPE-Bleo (ADR, vincristine, prednisone, etoposide and bleomycin) has been shown to be effective against relapsed Hodgkin's disease (Perren, 1990). Non-Hodgkin's lymphoma (NHL) also responds to ADR-containing combinations such as CHOP (cyclophosphamide, ADR [hydroxydaunomycin], vincristine [Oncovin], prednisone), which has activity against nodular and diffuse disease (Young, 1981). It should be noted that, for this disease, equivalent survivals have been obtained using protocols without ADR and with other, single agents (Portlock, 1980). In the case of childhood NHL, CHOP (9 weeks) or COMP (6 months) each followed by oral mercaptopurine and oral methotrexate (6 months) are treatments of choice, having approximately 90% survival rates. Nachman (1990) considers CHOP preferable because of its shorter duration. Diffuse large-cell lymphoma (DLCL) also responds to ADR-containing regimens, although some studies report equivalent survivals without it (Fisher, 1977; Skarin, 1980; Fisher, 1980). In contrast, one study which used multivariate

statistics to compare CHOP, [M]BACOD (methotrexate, bleomycin, Adriamycin, cyclophosphamide, vincristine, and dexamethasone) and MACOP-B (prednisone substituted for dexamethasone) against DLCL found ADR dose intensity to be the most significant predictor of response (Kwak, 1990).

ADR is the most effective single agent against previously untreated breast cancer (43% response; 39% partial, 4% complete) (Tormey, 1977), although better responses are obtained with various ADR-containing regimens (55-86%) (Tormey, 1977; Davis, 1978). One study using CAF (cyclophosphamide, ADR, flurouracil) against breast cancer produced a response in 82% of patients compared to 62% for a protocol with methotrexate substituted for ADR (Bull, 1978). In a ten year follow-up study on 222 patients treated with mastectomy, post-operative radiotherapy and FAC (flurouracil, ADR, cyclophosphamide) Buzdar reports 62% and 40% survival rates of phase II and III patients (1989). Another compared DAVH (dibromodulcitol, ADR, vincristine, Halotestin) with TAVH (thiotepa substituted for dibromodulcitol) and found less frequent toxic side effects with TAVH as well as significantly better response (39% vs. 24%) to TAVH among patients who had failed within 4 months of prior adjuvant chemotherapy (Skeel, 1989). Patel et al. (1989) report the use of FAC on ten male breast cancer patients.

ADR shows no significant gains over other single agents or combinations in treatment of ovarian cancer; it should be noted in particular that ADR alone shows poor response after other agents have failed to cure this condition (Young, 1981). Watring (1989) reports that six courses of PAC (platinum, ADR, Cytoxan) against ovarian cancer have a higher therapeutic index than nine courses as the survival rates are equivalent, but toxicity is higher after nine courses. Lawton (1990) recommends alternating platinum-containing regimens

with non-platinum, ADR-containing cycles to reduce toxicity. Hardy (1989) reported an 11% incidence of cerebral metastases after PAC was used against ovarian cancer and recommends CNS radiotherapy with PAC. Ozols (1987) used ADR (50 mg/m^2) with verapamil against refractory ovarian cancer, but found unacceptable cardiotoxicity in addition to no objective response.

ADR shows activity against osteogenic sarcoma, although one study concluded that ADR provided no significant advantage over resection alone (Gilchrist, 1978). ADR (70 mg/m^2) given every 3-4 weeks is the most effective single agent against soft tissue sarcoma and is a component of the most effective combination, CYVADIC (cyclophosphamide, vincristine, ADR, dacarbazine) with an average response of 40% (Young, 1981). One group attempted to use EAP (etoposide, ADR, cisplatin) against advanced gastric cancer, but were hindered by a severe myelotoxicity. One patient of ten had a partial response of less than 3.5 months (Sparano, 1990). ADR has shown activity against several other tumor types including metastatic adenocarcinoma, carcinoma of the bladder, bronchogenic carcinoma, neuroblastoma, metastatic thyroid carcinoma, and several lung cancers: small cell carcinoma, non-small cell carcinoma, and malignant mesothelioma (Young, 1981).

Limitations to use

ADR has a narrow therapeutic index, as well as a steep dose response curve (Frei, 1980), necessitating close dosage monitoring and reduction of dose in cases of hyperbilirubinaemia (Benjamin, 1974). It is subject to many of the acute toxicities associated with other antitumor agents: myelosuppression,

alopecia, mucositis, GI distress, and is additionally subject to an unusual delayed cardiotoxicity (Speth, 1988).

The cardiotoxicity is cumulative and dose-dependent; a very low (<1%) incidence is seen below 550 mg/m² (14). This iatrogenic irreversible cardiotoxicity is described as similar to dilated cardiomyopathy (Lefrak, 1973). At the microscopic level, swelling of the sarcoplasmic reticulum, vacuolization of the mitochondria and loss of heart muscle myofibrils are seen (Billingham, 1978). This phenomenon is documented in most mammals, including rats, in which studies have documented decreased α -actin isoform synthesis and decreased contractility in response to ADR (Lewis, 1987). Several hypotheses have been put forth to explain this toxicity, although none have been proven. One hypothesis implicates the covalent binding of cardiolipin (a mitochondrial phospholipid) by ADR (see Mechanisms of Action) (Tritton, 1978). This mechanism has been demonstrated *in vitro*. Using isolated heart muscle, other researchers have demonstrated ADR-related inhibition of cardiac enzymes NADP-linked isocitrate dehydrogenase, malic enzyme 6-phosphogluconic dehydrogenase (Fabregat, 1984), and glutathione peroxidase (Doroshov, 1989, Katki, 1980). This enzyme inhibition can be prevented with thiol (-SH) protecting compounds (DTT, cysteine), supporting the hypothesis that ADR inactivates thiols on essential myocardial enzymes which cumulatively leads to the observed toxicity (Fabregat, 1984). One study has implicated the major adriamycin metabolite, adriamycinol, in cardiotoxicity (Olson, et al., 1988).

Another hypothesis implicates O₂^{•-} formation in cardiotoxicity. Increasing evidence indicates that this may be the most significant cause of ADR-induced cardiotoxicity (Young, 1981). The proposed reason for the heart's susceptibility to this mechanism is two-fold: the heart probably has elevated

production of superoxide radicals and seems to have decreased elimination of such radicals (Young, 1981). An increase in radical production is likely because ADR stimulated superoxide production in mitochondria has been demonstrated; the heart is relatively enriched in mitochondria (Thayer, 1977). Normally, cardiac tissues handle superoxide by conversion to H_2O_2 via superoxide dismutase with subsequent inactivation by glutathione peroxidase (Doroshov, 1980; Katki, 1980). Unfortunately, cardiac glutathione peroxidase is one of the enzymes inhibited by ADR, as discussed above.

ADR is also subject to an acute cardiac toxicity characterized by hypereosinophilia of myocytes, focal necrosis, and polymorphonuclear leukocyte infiltration (Bristow et al., 1983). This response can be prevented *in vitro* by sodium cromoglycate, theophylline, or polyethylene glycol 400 and is thought to be the result of histamine release (Decorti et al., 1989). Bristow et al. (1983) report that histamine produced an effect on cardiac morphology almost identical to Adriamycin.

Mechanisms of action

Several mechanisms of action have been demonstrated for ADR. Tumor killing and toxicity to normal tissues may be the result of a combination of these (Speth, 1988). ADR intercalates DNA and binds to it, causing inhibition of DNA replication, transcription of DNA into RNA and protein synthesis (Painter, 1978). The finding that the potent antineoplastic ADR analogs, carminomycin and marcellomycin, inhibit only RNA synthesis, not DNA synthesis, indicates that RNA synthesis inhibition may be the more important mechanism of ADR (Daskal, 1978). ADR has also been shown to interact with

DNA topoisomerase II resulting in single and double stranded breaks and sister chromatid exchange (Tewey, 1984; Ross, 1981). These lesions are probably the result of stabilization of the so-called "cleavable complex" which is an intermediate step in normal topoisomerase II function (Bodley, 1988; Chen, 1986). As a result, ADR is both carcinogenic and mutagenic (Young, 1981). The nature of these mechanisms is such that they are significant only against actively dividing cells.

ADR damages membranes in at least three ways (Young, 1981; Speth, 1988). First, ADR causes clustering of cardiolipin, a membrane lipid found at increased levels in tumor cells and cardiac mitochondria (Tritton, 1978). Second, the hydrophobicity of the naphthoquinone group allows ADR to partition in the lipid phase of the lipid bilayer which alters membrane fluidity (Murphree, 1981). In a related issue, it has been observed that decreased membrane fluidity can result in ADR resistance (Siegfried, 1983). Finally, ADR undergoes reduction-oxidation cycling, thereby generating free radicals which cause lipid peroxidation. The mechanism for this is shown (Fig. 2) The enzyme P450 reductase reduces ADR, leaving a semiquinone radical. This radical then reduces O_2 to $O_2^{\cdot-}$ (superoxide ion). Superoxide ion can react with lipids, and any other cell constituent or it can participate in the production of $HO^{\cdot}$ which, in turn, reacts with virtually any cell constituent.

ADR also inhibits several metabolic pathways in a non-cell-cycle-specific manner. These include Na/K ATPase (Gosalvez, 1979), oxidative phosphorylation in mitochondria (Mailer, 1976; Kishi, 1976), NADP-linked isocitrate dehydrogenase (Yasumi, 1980), transmembrane ferricyanide reductase (McLoughlin, 1984), and plasma membrane NADH dehydrogenase (Sun, 1984).

Distribution Kinetics

When injected intravenously, ADR rapidly distributes to tissues. In humans, distribution half-life is consistently in the range of 4 min. (.07 hr) (Robert, 1987; Gil, 1983; Robert, 1982; Peterson, 1986). Reports of volumes of distribution in the central compartment (V_c) in humans are less consistent, ranging from <20 L/kg to 30 L/kg (Greene, 1983; Oosterbaan, 1983; Speth, 1987). Area under the plasma concentration versus time curve (AUC) is a dose-dependent parameter and, therefore, varies somewhat with the dose used in a given study. At commonly used doses (25-50 mg/m²) AUC's range approximately between 0.7 and 2.0 $\mu\text{g}\cdot\text{hr}/\text{ml}$ (Eksborg, 1985; Eksborg, 1985b, Gil, 1983).

Distribution kinetics have been examined in several non-human species. One study performed in the rat reported distribution half-life to be 0.09 hr (Rahman, 1986). That study also reported an AUC of 1.95 after rats were administered 6 mg/kg ADR. Other studies in dogs and rabbits report distribution half-lives of 0.09 (Oosterbaan, 1982) and 0.02 hr (Mimnaugh, 1978), respectively. V_c are reasonably consistent among species: 1.8 L/kg-rat (Rahman, 1986), 1.5 L/kg-dog (Oosterbaan, 1982), 0.7 L/kg-rabbit (Eksborg, 1985b).

Metabolism

An understanding of ADR metabolism is crucial because it appears that much of the pharmacodynamics, as well as toxic side effects, is linked to metabolism. ADR is biotransformed by a cytoplasmic NADPH-dependent

oxidoreductase (EC 1.1.1.2) to adriamycinol (Felsted, 1977, Turner, 1976), and by microsomal NADPH-cytochrome P₄₅₀ reductase 7-deoxyaglycones (Oki, 1977) (Fig. 1). Aglycones are thought to be a minor pathway that results from reaction with HO[•] produced by adriamycin's redox cycling (Fig. 2) (Schwartz, 1984). Adriamycinol retains the ability to intercalate DNA and, therefore, retains cytotoxicity (Bachur, 1976; Meriweather, 1971). It is the only metabolite thought to retain cytotoxicity. All three classes of metabolites have been documented in the humans, dogs, rats, mice, rabbits (Loveless, 1978), and hamsters (Bachur, 1973).

ADR is the predominant form found in plasma and all tissues except for brain, liver, and rat heart, where adriamycinol is most abundant (Loveless, 1978). Liver and kidney have the highest glycosidase activity and, therefore, have higher concentrations of aglycone metabolites (Loveless, 1978). Studies using isolated rat and rabbit hepatocytes report that glucuronidation of ADR metabolites proceeds slowly and is probably the rate-limiting step in the excretion of the lipophilic aglycones and 7-deoxyaglycones (Bachur, 1973, Bachur, 1971).

Elimination

ADR is eliminated primarily in the bile, although there is some urinary excretion. In the rabbit, 17% of injected ADR is excreted in the bile by 8 hr; 30% of this is ADR, 60% is adriamycinol, 3% is aglycone, and 7% is polar conjugates (presumably glucuronides) (Bachur, 1974). In this period only 2% is excreted in the urine; 60% of this is ADR, 35% is adriamycinol, <5% is polar conjugates (Bachur, 1974). Excretion in human urine seems slightly different

with ADR and adriamycinol accounting for 39% and 28%, respectively (Takanashi, 1976). No data are available for human biliary excretion.

It is difficult to compare the elimination half-lives reported in human studies because some studies use two-compartment models while others use three-compartment models to make the calculation. Most human studies have used a three compartment model ($C_t = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$, where C_t is plasma concentration at time t ; A , α , B , β and C , γ are intercepts and slopes of the distribution, postdistribution and elimination phases, respectively) where the elimination half-life ranges between 10 and 30 hours (Benjamin, 1977, Eksborg, 1985b, Oosterbaan, 1982). Where elimination half-lives are reported for the dog and the rat, they also fall within this range (Oosterbaan, 1984, Rahman, 1986). Clearance of ADR seems to vary consistently among species; that is, smaller species tend to have higher clearance rates. For example, human clearance falls in the 10-20 ml/min/kg range while the dog, rabbit and rat have clearances of about 50 ml/min/kg (Oosterbaan, 1984), 40 ml/min/kg (Bachur, 1974), and 44 ml/min/kg (Rahman, 1986), respectively.

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APPENDIX

FIGURES

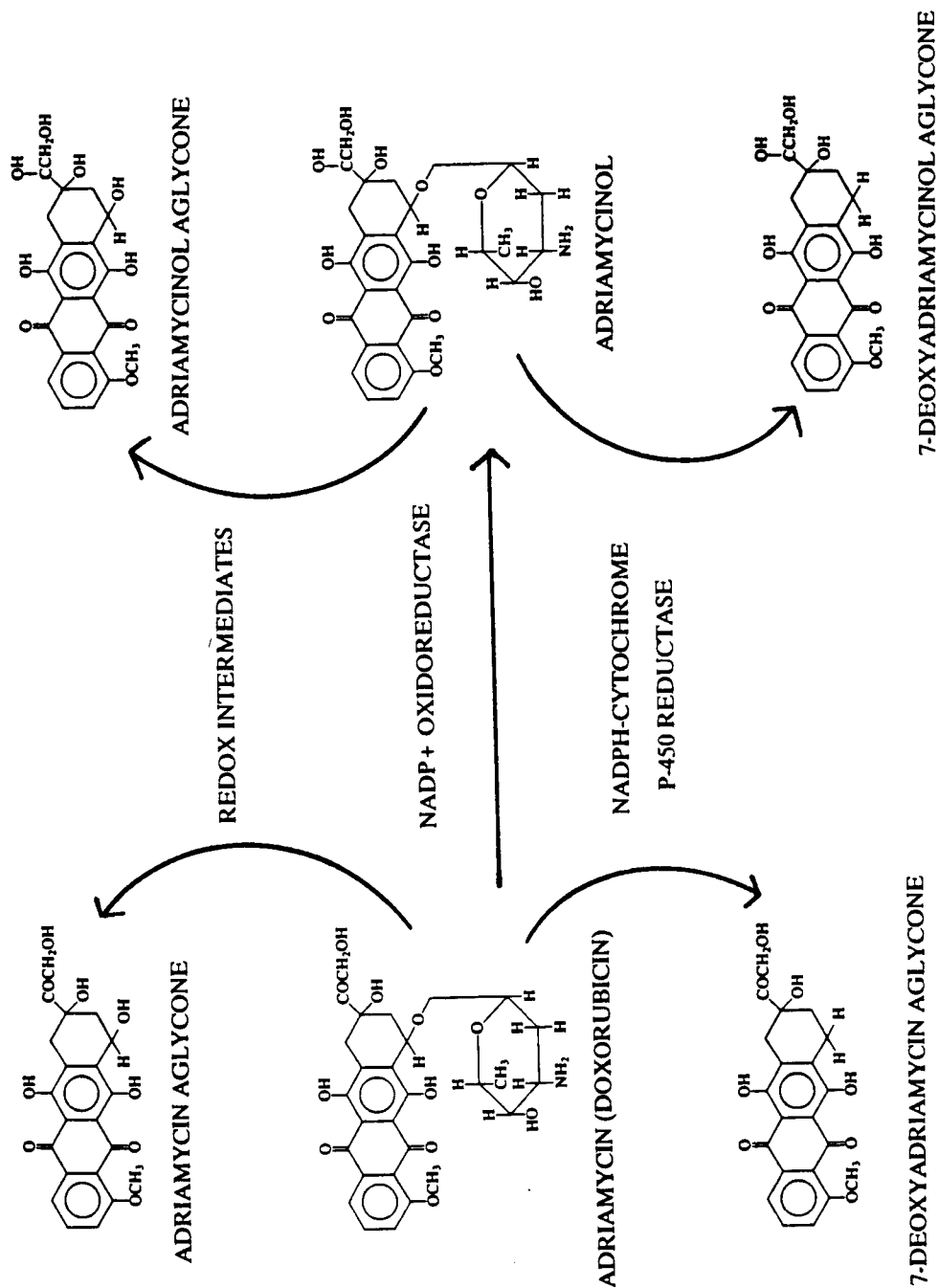


Fig. 1 Adriamycin and its major metabolites.

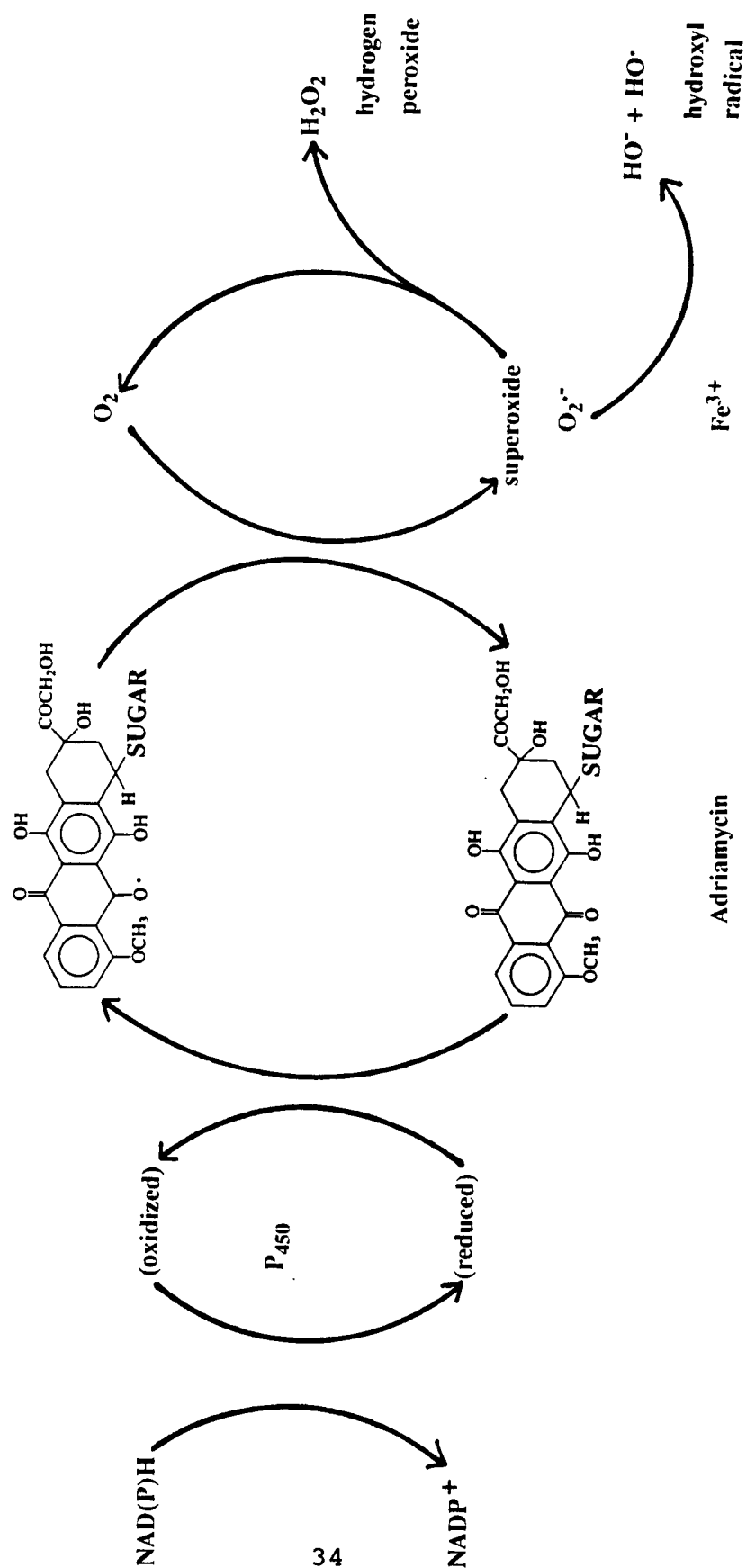


Figure 2. Adriamycin redox cycling.

PART 2

THE EFFECT OF HYPERTHERMIA ON NORMAL TISSUE TOXICITY AND ON ADRIAMYCIN PHARMACOKINETICS IN DOGS

CHAPTER 1: INTRODUCTION

Hyperthermia has been shown to potentiate cytotoxicity of several anti-tumor drugs including the anthracycline antibiotic, Adriamycin (Hahn, 1979; Herman et al., 1982; Herman, 1982). At the cellular level, hyperthermic conditions lead to an increased influx of Adriamycin into cells and increased cell kill (Rice and Hahn, 1987). The *in vivo* situation is clearly more complex as it includes additional factors; most notably, drug delivery to a given tissue must be considered.

Milligan has reported that whole body hyperthermia increased blood flow to the gastrointestinal tract, heart, and pancreas (Milligan et al., in press). The phenomenon of increased drug delivery to the gastrointestinal tract is supported by a report of bloody diarrhea in dogs after concurrent whole body hyperthermia and Adriamycin (Daly et al., 1984). Other studies have reported nonuniform drug delivery with concurrent whole body hyperthermia (Riviere et al., 1990).

The purpose of this study was to determine whether whole body hyperthermia altered the pharmacokinetics of Adriamycin and whether the concurrent administration of whole body hyperthermia and Adriamycin increased gastrointestinal toxicity.

CHAPTER 2: MATERIALS AND METHODS

Adult pure-bred beagle dogs of both sexes (Marshall Farms, North Rose, NY) were divided into 3 treatment groups (n=5): ADR, intravenous Adriamycin-HCl (Adria Labs, Columbus, OH) 30 mg/m²; WBH, whole-body hyperthermia 42°C for 1 hr.; ADR + WBH, 42°C hyperthermia for 1 hour, with Adriamycin administered when rectal temperature reached 42°C. 30 mg/m² ADR is the standard clinical dose for canine patients (Ogilvie et al., 1989); higher doses (50-60 mg/m²) are often used in humans (Speth et al., 1987; Gil et al., 1983; Robert et al., 1987), but are not tolerated by dogs. Dogs were fasted overnight before treatment, but were given free access to water. In all groups, dogs were premedicated with butorphanol (0.4 mg/lb.), diazepam (0.2 mg/lb.) and atropine (0.04 mg/lb.); anesthesia was mask-induced and maintained with isoflurane. 42°C WBH was induced in appropriate groups with a thermal blanket that utilizes recirculating heated water. Temperature was monitored using a Bailey TM-10A with internal calibration and intraperitoneal, intramuscular, and rectal thermocouples (one probe per site). Rectal temperature was used as the primary indicator for monitoring purposes; approximately one hour was needed to raise rectal temperature to 42°C.

Adriamycin Pharmacokinetics

In the ADR and ADR + WBH groups, venous blood (3 ml) was collected immediately, at 15, 30, 45 minutes, 1, 2, 4, and 6 hours after Adriamycin administration. Duodenal biopsies were collected via endoscopy immediately after and 1 hour after drug administration for measurement of ADR tissue

concentrations. All samples were placed on ice immediately, blood was then centrifuged and the plasma and biopsies were stored at -20°C until analysis.

Plasma extraction of ADR and its metabolites was carried out using a modification of Robert's method (Robert, 1980). Briefly, daunomycin (DMN) was added as internal standard to 1 ml plasma and the sample was purified using C-18 Sep-Pak minicolumns (Waters Assoc., Milford, MA) with phosphate buffer and methanol. Duodenal biopsies were homogenized with a Dounce tissue grinder in 1 ml phosphate buffer and then treated like plasma (Cox et al., 1991).

The HPLC system consisted of a μ -Bondapak phenyl (10 cm X 80 mm) column protected by a CN-type Guardpak (Waters Assoc.) column. The mobile phase consisted of a gradient of 0.1% ammonium formate (pH 4.0) and acetonitrile. Fluorescence of the eluate was monitored with excitation at 480 nm and emission at 550 nm (Waters Model 470 Detector).

Pharmacokinetic parameters were determined using an automated curve-stripping program followed by a SAS-based nonlinear regression program. Calculation of parameters shown in Table 1 were as follows:

Cp_0 = extrapolated drug concentration in the central compartment at time = 0

$\text{VD}_c = \text{Dose}/\text{Cp}_0$

$\text{VD}_\beta = \text{Dose}/\beta * \text{AUC}$, where β is the slope of the elimination phase

$\text{VD}_{\text{area}} = \text{Dose}/K * \text{AUC}$, where K is the first order elimination rate constant

$\text{Cl} = \text{Dose}/\text{AUC}$

$t_{1/2\alpha} = \ln 2/\alpha$, where α is the slope of the distribution phase

$t_{1/2\beta} = \ln 2/\beta$, where β is the slope of the elimination phase

Area under the plasma concentration versus time curve (AUC) was calculated by the trapezoidal method (Gibaldi and Perrier, 1982). VD_c , VD_β , and VD_{area} represent volumes of distribution of the central compartment, elimination phase

and area, respectively. Cl is whole body clearance; $t_{1/2\alpha}$ and $t_{1/2\beta}$ are the half-lives in the distribution and elimination phases, respectively.

Statistical comparison of these parameters was performed using the Wilcoxon test (SAS, Cary, NC).

Cell Turnover

Duodenal biopsies were collected via endoscopy prior to treatment and at the following times after treatment: 1 hr., day 3, and day 7. Six biopsies were collected at each time and placed in polypropylene microcentrifuge tubes (2 biopsies per tube) containing 1 ml ice-cold Krebs-Ringer bicarbonate/glucose buffer. Procaine penicillin G (2000 units/ml), dihydrostreptomycin (0.25 mg/ml) and gentamicin (0.05 mg/ml) were added to eliminate bacterial growth. 0.5 μ Ci of ^{3} H-thymidine (ICN Biomedicals, Costa Mesa, CA) was added and the open vials were placed in a 37°C water bath for 1 hr. The atmosphere of the bath was maintained at 5%CO₂/95%O₂.

At the end of the incubation, DNA was extracted with perchloric acid and quantified with diphenylamine as described by Leyva and Kelley (1974). ^{3} H-thymidine uptake was quantified by mixing aliquots of the acid extract with Pico-Flor 40 (Packard, Downers Grove, IL) and counting (Packard Liquid Scintillation Analyzer Model 1500).

Histopathology

Duodenal biopsies were collected for histopathological examination prior to treatment and on Day 3 following treatment. Biopsies were fixed in neutral

buffered formalin prior to sectioning, hemotoxylin-eosin staining and light microscopic examination.

Enzyme Assays

Duodenal biopsies were collected prior to treatment and on Day 3 following treatment for assay of gamma-glutamyl transpeptidase (GGT), N-acetyl- β -D-glucosaminidase (NAG), and succinyl dehydrogenase (SDH). These assays were performed according to the micromethods developed by Andersen, et al. (1983). When necessary, multiple biopsies at a given time were combined to give a sample size of at least 5 mg. Briefly, samples were homogenized in 1 ml of ice-cold 0.15 M NaCl, pH 7.0, with a Dounce tissue grinder (Wheaton, Millville, NJ). Protein concentration of this homogenate was determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). GGT and NAG assays consisted of incubation of homogenate aliquots with the fluorescently-labelled substrate analogs, N-gamma-glutamyl- α -naphthylamide and 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside, respectively. At 40 and 60 min. for GGT and NAG, respectively, the reactions were stopped and liberated fluorescent label quantified using a Perkin-Elmer LS5B fluorimeter. The SDH assay is based on reduction of p-iodonitrotetrazolium (INT) to its UV-absorbing form. INT-formazan was then extracted with ethyl acetate and quantified using a Shimadzu UV-Visible spectrophotometer (490 nm).

CHAPTER 3: RESULTS

Pharmacokinetics

The HPLC system used in this study is capable of separating DMN (internal standard), ADR, and the metabolites of ADR, adriamycinol (ADR-OL), ADR aglycone, and ADR-OL aglycone. ADR-OL was the only metabolite detected in significant quantities in the plasma; however, its appearance varied between dogs. Whole body hyperthermia decreased peak plasma concentration of ADR-OL ($0.0126 \pm 0.0016 \mu\text{g/mL} \pm \text{SEM}$ vs. 0.573 ± 0.020 ; $p=0.09$). Representative plasma concentration versus time profiles are shown (Fig. 1). ADR aglycone and ADR-OL aglycone (both $< 5 \text{ ng/ml}$) were seen in 4 animals (2 ADR, 2 ADR + WBH). ADR aglycone was detected no later than 45 minutes in the ADR group and no later than 5 minutes after drug administration in the ADR + WBH group. ADR-OL aglycone was detected up to 30 minutes after drug administration in the ADR group and up to 15 minutes after drug administration in the ADR + WBH group.

Plasma elimination of ADR was best described by a 2 compartment model (Fig. 2) characterized by a rapid distribution phase ($t_{1/2\alpha}=0.07 \text{ hr}$) and a slower elimination phase ($t_{1/2\beta}=1.34 \text{ hr}$). WBH induced significant changes in clearance (Cl), apparent volumes of distribution (VD_c , VD_β , VD_{area}) and area under the plasma concentration versus time curve (AUC) (Table 1).

ADR concentrations in duodenal samples did not differ significantly between the two drug treatment groups. WBH caused higher ADR concentrations initially followed by more rapid elimination of ADR from the

duodenal epithelium (Fig. 3), however, these differences were not statistically significant.

Cell Turnover

Cell turnover data showed considerable variability. Although comparisons of mean cell turnover values at specific time points to pretreatment control values were not statistically significant, some trends were seen (Fig. 4). ^3H -thymidine uptake in drug-containing groups (ADR, ADR + WBH) reached a maximum depression on day 3 following treatment whereas the WBH group values were most depressed on the day of treatment. The two drug-containing groups had similar responses; their absolute differences were probably due to the higher pretreatment values of the ADR + WBH group.

Histopathology and Clinical Parameters

Duodenal biopsies from each treatment group were histopathologically normal. Complete blood counts and chemistry panels were normal; mean values within groups were never outside the clinically normal range. Creatinine clearance was significantly reduced on day 3 compared to pretreatment clearance (4.04 vs. 2.67 ml/min*kg) in the ADR + WBH group. Posttreatment creatinine clearance was within normal limits for the ADR and WBH treatment groups.

Enzyme Assays

Activities for the enzymes GGT, NAG, and SDH were not statistically changed by the treatment protocols when day 3 samples were compared to pretreatment (Table 2). Posttreatment GGT activity was increased slightly in the WBH and ADR + WBH treatment groups whereas the ADR group was relatively unchanged.

CHAPTER 4: DISCUSSION

Altered plasma or tissue concentrations of Adriamycin or its metabolites resulting from concurrent administration of whole body hyperthermia may affect toxicity towards cancer cells as well as normal tissues. Only Adriamycin and adriamycinol have been previously described in the dog (Oosterbaan et al., 1984). In the present study, the HPLC system used was capable of separating Adriamycin, adriamycinol and the respective aglycones. All four compounds were detected in plasma; no metabolites were detected in duodenal biopsies. The alcohol derivative of Adriamycin, ADR-OL, probably does not have significant anti-tumor activity, but has been implicated in Adriamycin-induced cardiotoxicity (Olson et al., 1988). Increased cardiotoxicity in humans receiving concurrent whole body hyperthermia and Adriamycin has been reported (Kim et al., 1979). The present study did not examine ADR-OL content of cardiac tissues, however, our results indicate that peak ADR-OL plasma concentrations were decreased by hyperthermia.

This study demonstrated that whole body hyperthermia altered the pharmacokinetics of Adriamycin in the normal dog. Specifically, increases in the rate of drug distribution to tissue and the volume of distribution of drug occurred. An increase in whole body clearance, a decrease in AUC and a trend toward decreased peak plasma concentration (C_{p0}) were noted. These increases in tissue distribution did not seem to result in overt toxicity to the duodenum. Although changes in other parameters are seen (e.g., B , β , and VD_c), these changes are not statistically significant probably due to small sample size and variability within groups.

Various investigators have reported different results for Adriamycin pharmacokinetics in response to whole body hyperthermia. Daly, et al. (1987) reported no change in mean Adriamycin plasma concentrations during 43°C hyperthermia induced by extracorporeal heating of blood in heparinized dogs. Animals were given a dose (2 mg/kg) comparable to the dose used in the present study, but spectrofluorometric methods were used and pharmacokinetic parameters were not calculated for the four animals used. Daly's study reported bloody diarrhea lasting for 7 days after treatment which was attributed to increased drug levels in the gastrointestinal tissues. Mimnaugh et al. (1978), reported that WBH affected only the initial rate of Adriamycin distribution to tissues ($t_{1/2\alpha}$) in rabbits and not the volume of distribution. This conflicts with our finding of increased volumes of distribution of Adriamycin with concurrent hyperthermia. Mimnaugh also reported that whole body hyperthermia did not increase Adriamycin concentrations in tissue samples (including duodenum) collected 60 minutes after treatment, with the exception of cardiac muscle. Our finding of a tendency toward decreased peak ADR plasma concentration (Cp_0) with WBH also does not agree with Mimnaugh's significantly increased Cp_0 in heated rabbits. Cp_0 may be an important parameter in light of reports that peak Adriamycin concentrations are directly related to incidence of toxicity (Speth et al., 1987; Legha et al., 1982; Vogelzany, 1984). The results of the present study are similar to the changes in the pharmacokinetic disposition of cisplatin when administered concurrently with whole body hyperthermia to dogs (Daly et al., 1984). The differences in pharmacokinetics may be attributable to different physiologic responses to hyperthermia in the two species.

Ex vivo ^3H -thymidine incorporation has been used to study the effects of drugs on intestinal mucosa proliferative activity (Craven et al., 1988). In the

present study, a single dose of Adriamycin decreased proliferative activity in the duodenal mucosa for at least two days; however, activity returned to normal by one week. A pilot study evaluating cell turnover on days 1-7 showed maximum depression of activity on day 3; therefore, dogs in this study were biopsied only on days 1, 3, and 7 to minimize anesthesia and trauma to the gastrointestinal tract. Whole body hyperthermia alone produced little change in the proliferative activity of duodenal mucosa. The depression of proliferative activity seen in the groups receiving Adriamycin can be attributed to the well documented effects of Adriamycin on DNA replication systems (Bodely and Liu, 1988; Tewey et al., 1984; Zwelling et al., 1981; Chen and Liu, 1986). The effects include inhibition of DNA topoisomerase II and intercalation into DNA, both of which may interfere with DNA replication. The variability in this data may be a result of the inherent nonuniformity in biopsy size and depth. Differences in the number of villus tips versus crypt cells could be reflected in cell turnover rates. Additionally, ^3H -thymidine incorporation in larger biopsies may have been limited by diffusion of labelled thymidine into the entire biopsy.

The results of the enzyme assays indicated that the treatment protocols significantly affected neither GGT, NAG nor SDH activities. These enzymes are brush border, lysosomal, and mitochondrial enzymes, respectively (Andersen et al., 1983). The activities of these three enzymes have been shown to be altered by intestinal disease (Andersen et al., 1983). There seemed to be a slight induction of GGT in the hyperthermic groups which was not seen in the ADR group. The reason for this enzyme induction is not clear.

Significant changes in complete blood counts and serum chemistry parameters were not seen in the present study. These parameters would more likely be changed with repeated drug administration.

In conclusion, this study demonstrated that whole body hyperthermia increases the distribution of Adriamycin in the normal dog. There is not, however, a uniform increase to all tissues. It seems likely that blood flow to tumors in various tissues would differ just as it differs in various normal tissues. Whether the pharmacokinetic changes seen would increase or decrease Adriamycin concentrations in a spontaneous tumor and the effect on tumor cell killing would likely depend on the location of the tumor. Whole body hyperthermia also increased clearance of Adriamycin from plasma which may be a result of increased excretion as well as distribution. Since fractionated hyperthermia protocols are often used clinically, the effects of repeated heating on plasma and tissue pharmacokinetics and toxicity should be examined. Additionally, as blood flow patterns probably change during hyperthermia, the question of pharmacokinetics after continuous infusion with concurrent hyperthermia needs to be addressed.

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APPENDIX

TABLES AND FIGURES

Table 1. Pharmacokinetic parameters describing the disposition of adriamycin in normal dogs with and without concurrent whole body hyperthermia (42°C).

	ADR ^a	ADR + WBH	
A, µg/ml	5.82±0.990	2.76±1.35	
α, hr ⁻¹	11.3±1.08	15.0±2.69	
B, µg/ml	0.112±0.052	0.024±0.002	
β, hr ⁻¹	0.730±0.215	0.339±0.030	
VD _c , L/kg	0.281±0.059	1.32±0.569	
VD _β , L/kg	15.9±4.29	53.7±5.39	<i>b</i>
VD _{area} , L/kg	4.55±1.25	23.1±5.85	<i>b</i>
T _{1/2α} , hr	0.065±0.006	0.052±0.007	
T _{1/2β} , hr	1.34±0.41	2.09±0.153	
Cl, ml/kg/min	39.8±5.30	137.0±42.3	<i>b</i>
Cp ₀ , µg/ml	5.94±1.01	2.78±1.35	
AUC, µg/ml*hr	0.648±0.084	0.236±0.061	<i>b</i>

^a treatment groups (n=5 each); all values are mean±SE

^b p<.05, Mann-Whitney

Table 2. Enzyme activities of duodenal biopsies from normal dogs receiving adriamycin (ADR), 42°C whole body hyperthermia (WBH) for 1 hour or both concurrently (ADR + WBH). Pinch biopsies were collected via endoscopy. Assays were performed using either fluorescent labelled substrate analogs (GGT, NAG) or spectrophotometric methods (SDH).

	GGT ^b (units/g)	NAG (units/g)	SDH (10 ⁻³ units/g)
ADR ^a Pretreatment ^c	3.54±0.338	1.13±0.160	5.81±0.136
Day 3	3.49±0.269	0.984±0.105	4.13±0.213
WBH Pretreatment	2.92±0.220	1.23±0.183	4.17±0.775
Day 3	3.73±0.880	1.03±0.113	2.64±0.371
ADR Pretreatment	3.81±0.130	1.27±0.173	3.38±0.471
+ Day 3	4.40±0.338	1.32±0.187	2.93±0.195
WBH			

^a treatment groups (n=5 each); all values are mean±SE.

^b enzymes assayed: gamma-glutamyl transpeptidase (GGT), N-acetyl-β-D-glucosaminidase (NAG), succinyl dehydrogenase (SDH). Enzyme activities by treatment group were not significantly different (Mann-Whitney).

^c biopsy collection time relative to treatment.

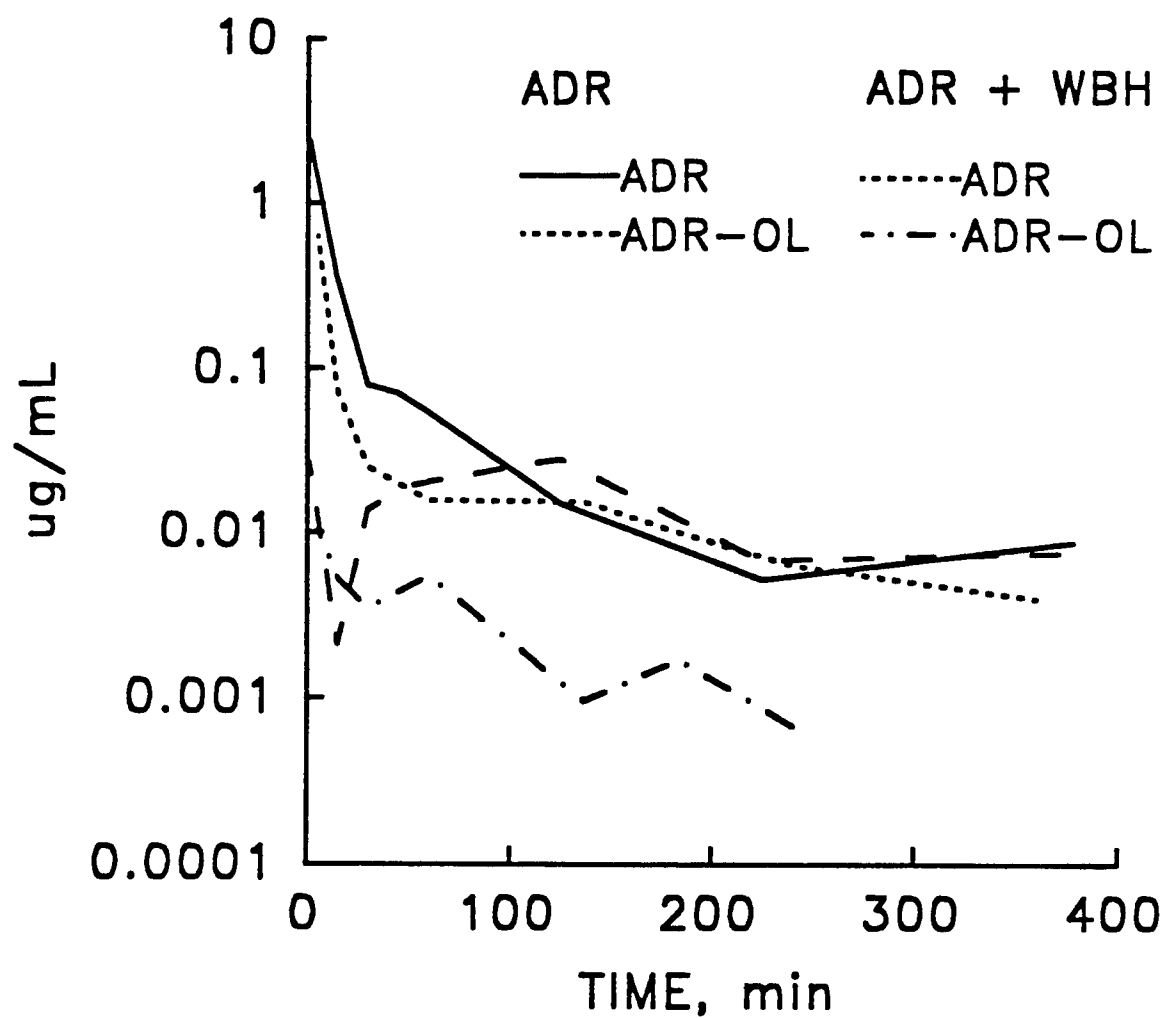


Figure 1. Representative plots of actual ADR and ADR-OL plasma concentrations in dogs receiving drug alone (ADR) and drug with concurrent whole body hyperthermia (ADR + WBH).

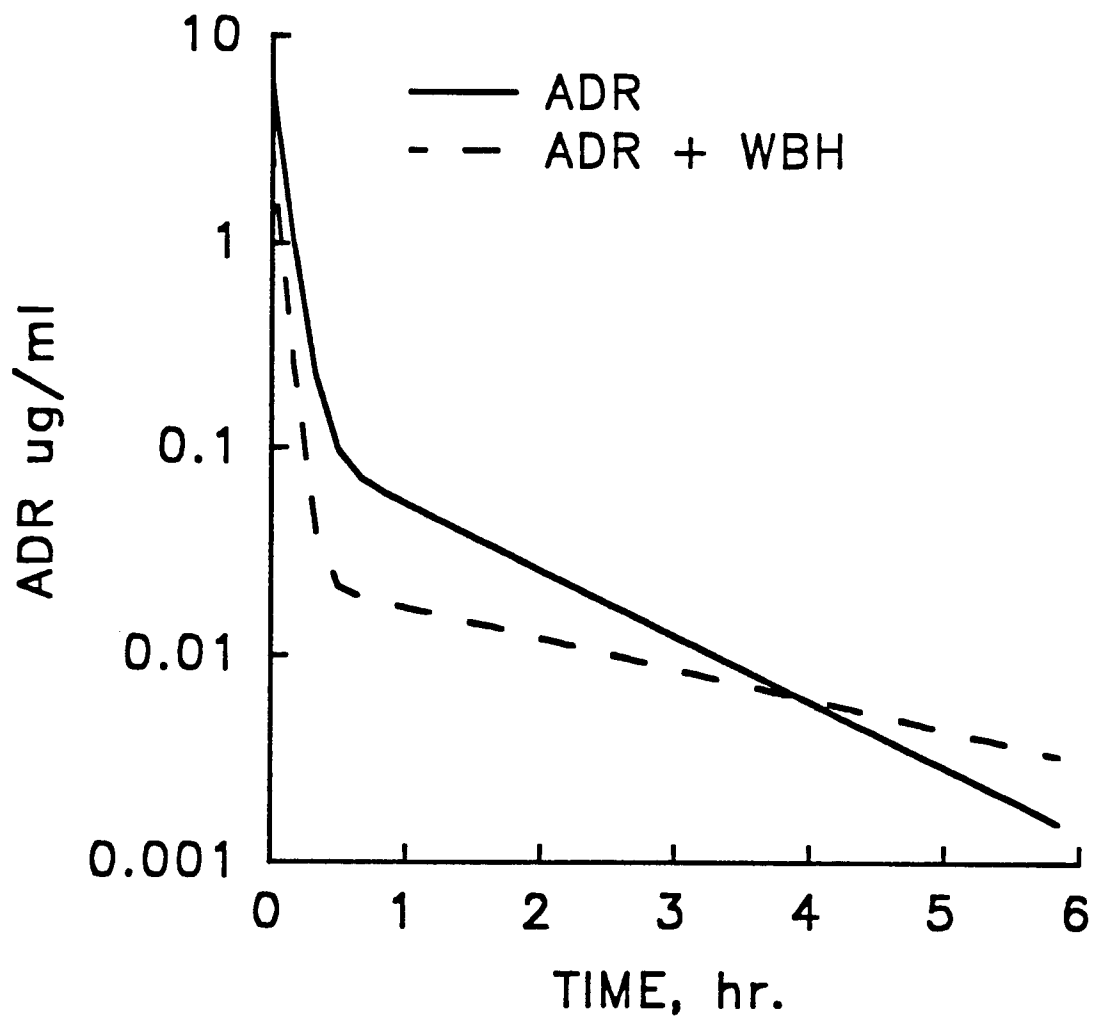


Figure 2. Plasma adriamycin concentration versus time curves generated from pharmacokinetic data using the formula:

$$C_{ADR} = A \cdot e^{-(\alpha \cdot t)} + B \cdot e^{-(\beta \cdot t)}.$$

A and B are intercepts, α and β are the slopes of the distribution and elimination phases, respectively and t is time since drug administration.

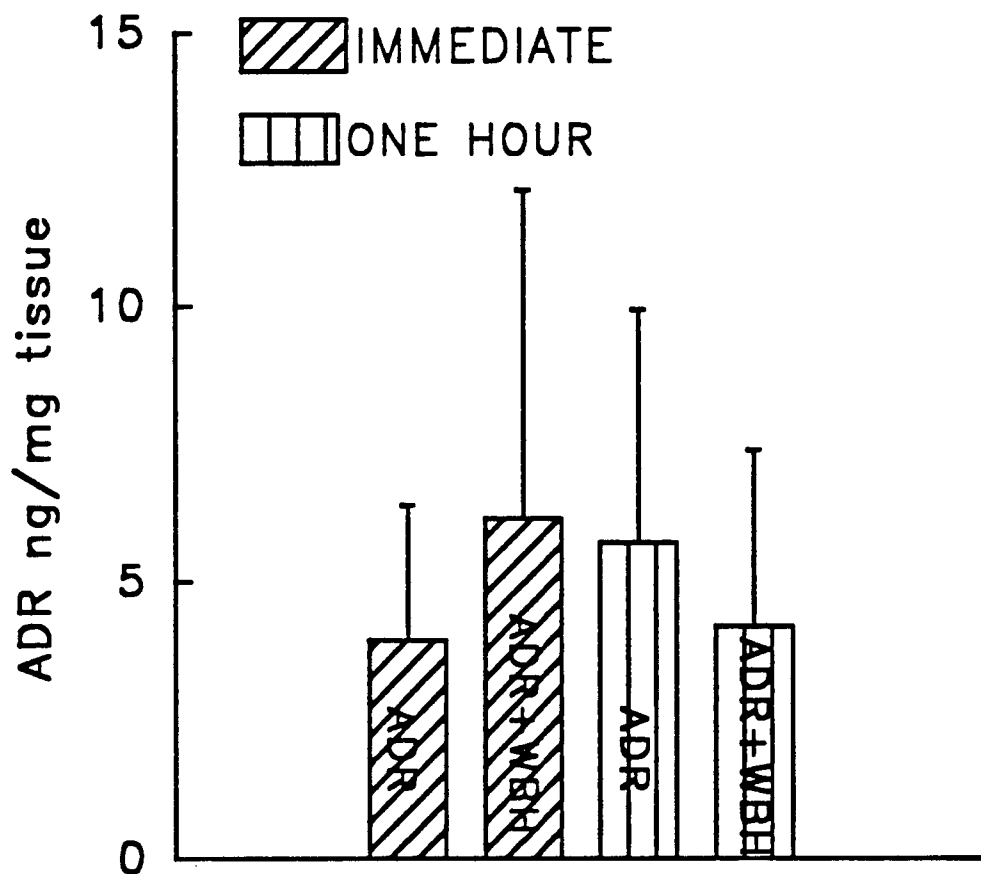


Figure 3. Adriamycin content of duodenal biopsies (mean $\pm$ SEM; n=5). Biopsies were collected immediately after and one hour after drug administration from dogs receiving drug alone (ADR) or drug concurrent with 42°C hyperthermia (ADR + WBH).

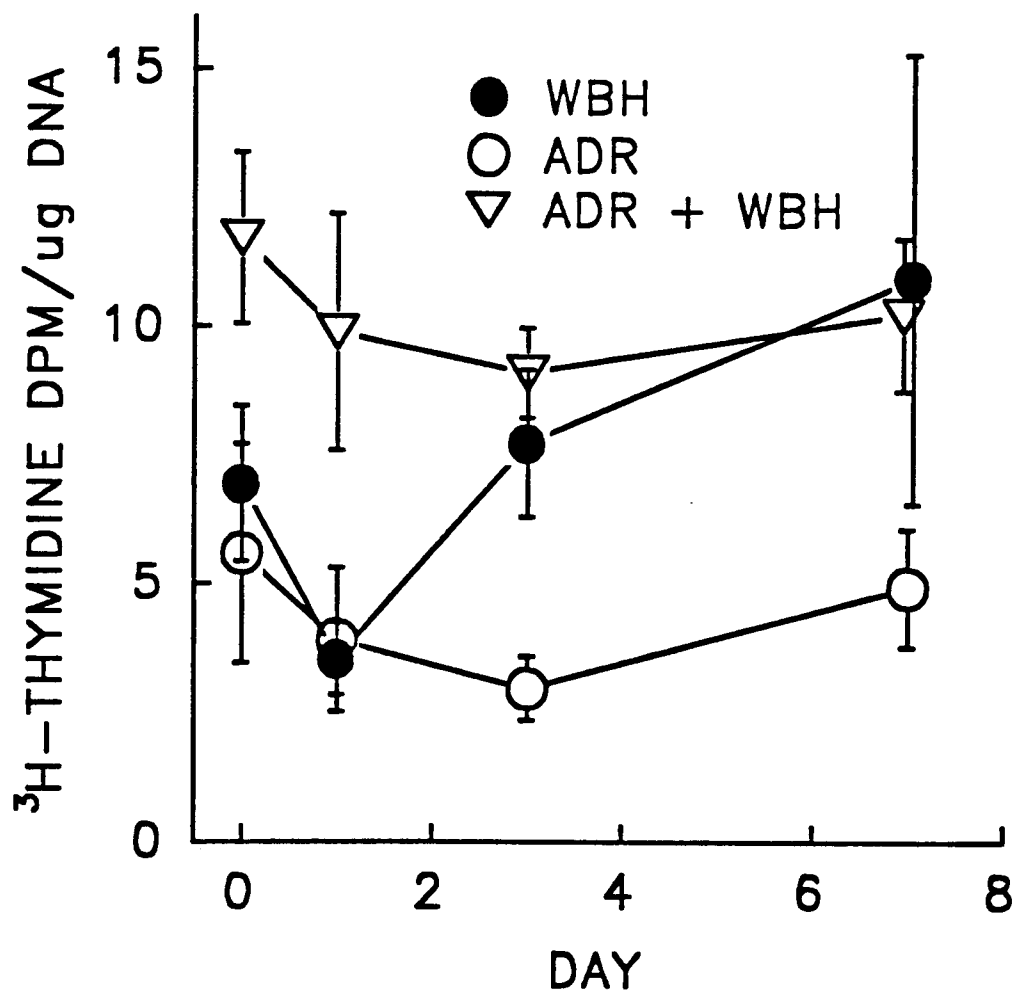


Figure 4. ^3H -Thymidine uptake by duodenal biopsies incubated for one hour in the presence of $0.5 \mu\text{Ci } ^3\text{H}$ -thymidine. (mean $\pm$ SEM; $n=5$)

PART 3

THE EFFECT OF HSP 70 EXPRESSION ON ADRIAMYCIN PHARMACOKINETICS IN AN *IN VIVO* RAT MAMMARY TUMOR MODEL

CHAPTER 1: INTRODUCTION

Adriamycin is an antineoplastic agent shown to have activity against several types of cancer, especially when used as part of a multi-drug protocol (Young, 1981). In an effort to extend its efficacy, several investigators have examined the combination of hyperthermia and Adriamycin, as well as several other antineoplastic agents (Engelhardt, 1987). This research was prompted, largely, by reports that this combination led to reduced survival of cells *in vitro* (Hahn et al., 1975). Results of studies on the *in vivo* use of Adriamycin with hyperthermia against tumors have been somewhat inconclusive (Engelhardt, 1987). Often these studies show no synergism or do so only at doses not tolerated by subjects (Hahn et al., 1975; Overgaard, 1976).

Additional *in vitro* studies demonstrated that, if hyperthermia was administered prior to Adriamycin, a protective effect was conferred by the hyperthermia (Hahn and Strande, 1976). It has been reported that cells made thermotolerant not only by heat, but also by ethanol are resistant to Adriamycin (Li and Hahn, 1978). Positive correlation between intracellular Adriamycin concentration and cytotoxicity has been reported (Rice and Hahn, 1987; Luk and Tannock, 1989). Also, it has been reported that, when synergism is present, it is largely due to increased influx of Adriamycin into cells (Rice and Hahn, 1987).

In view of the above observations, we proposed to determine whether prior local hyperthermia had any effect on Adriamycin concentrations in an *in vivo* tumor model. In addition, alteration in blood flow rates and their effect on drug concentrations were examined.

CHAPTER 2: MATERIALS AND METHODS

Tumor Model

50 day old female Sprague-Dawley rats were administered DMBA (10 mg/100 g) by gavage while under light halothane anesthesia. DMBA was dissolved in sesame oil (10 mg/ml). Mammary tumors were treated when they reached 1 cm in diameter, approximately 5 to 10 weeks later. Animals in all groups were euthanized using T-61 (0.2 cc, i.c.). Biopsies of all tumors were fixed in formalin prior to histologic examination and were confirmed as mammary adenocarcinomas after treatment.

Hyperthermia

Hyperthermia (43.5°C, 1 hr) was induced using a Nd:YAG (Surgical Laser Technologies, CL60, Tokyo, Japan) laser with accompanying forced air surface cooling on tumors of anesthetized rats (0.125 mg acepromazine maleate and 6.25 mg ketamine HCl, i.m.) as previously described (Panjepour et al., 1991). The laser was controlled by a K-type feedback probe placed near the base of the tumor. Three T-type probes in 24 ga. catheters were placed just under the skin, at the tumor center, and at the tumor base. Data was used only when the average temperature at the center of the probe was $43.5^{\circ}\text{C} \pm 5\%$.

HSP 70 Expression

The rate of HSP70 production was determined by injecting ^3H -methionine (200 uCi, i.v.) immediately at the end of 1 or 2 hyperthermia treatments (24 hr apart). The anesthetic protocol induced low levels of HSP70, so an unheated control group received only the standard anesthetic 1 hr before ^3H -methionine. In all three groups, rats were euthanized 3 hr after radiolabel and tumors were frozen.

SDS-PAGE was performed on tumors following the method of Fujio et al. (1987). Tumors were homogenized in 2 ml 10 mM NaH_2PO_4 , pH 7.2 that was 0.5 mM in phenylmethylsulfonyl fluoride, then centrifuged 15,000 X g for 15 min at 4°C. The first supernatant was then centrifuged 105,000g for 1 hr at 4°C. 100 μl second supernatant was diluted with 400 μl SDS sample buffer and boiled for 5 min. 10 μl of this mixture was then loaded onto each of 9 lanes of a 4% stacking/12% resolving SDS-polyacrylamide gel (mini 2-D, Bio-Rad, Costa Mesa, CA, U.S.A.) and electrophoresed at 200 volts for 45 min. Gels were silver stained (Bio-Rad, Costa Mesa, CA, U.S.A.) and photographed. The 70 kDa bands were cut from the gel, minced, and extracted at 50°C for 3 hr (Solvable, Du Pont, Boston, MA, U.S.A.). Liquid scintillation cocktail (Formula 989, Du Pont, Boston, MA, U.S.A.) was added prior to counting.

Blood Flow

Blood flow to tumors was determined using a modification of the reference sample technique of Malik (1976). Briefly, the left internal iliac and left ventricle via the right common carotid were cannulated with PE 10 tubing.

15.5 μ l ^{113}Sn microspheres in 10% Dextran, 0.01% Tween 80 (New England Nuclear, DuPont, Boston, MA, U.S.A.) were used. ^{113}Sn microspheres (0.46 ml, 12,000 spheres) were infused over a 10 second period into the left ventricle via the cannula. A withdrawal/infusion pump (Sage Instruments, Model 351, Orion Res. Inc., Boston MA, U.S.A.) simultaneously began to withdraw (0.3 ml/min for 70 sec) at the iliac cannula. Animals were euthanized with T-61 via the carotid cannula. Radioactivity in tissues was quantified using a gamma counter.

Adriamycin

Rats received Adriamycin (5 mg/kg, i.v., Adria Labs, Columbus, OH, U.S.A.) 3 hr after the end of 1 or 2 hyperthermia treatments to correspond the ^3H -methionine labelling period. An unheated control group received only the anesthetic 4 hours prior to Adriamycin. Blood was collected immediately after, 30 min after and 60 min (when applicable) after drug administration. Rats were euthanized at either 30 min or 60 min after dosing. A small piece of the tumor was analyzed immediately using FACS, the remainder was frozen for HPLC.

Tumor cell suspension were prepared for FACS by mincing a small piece of tumor in 3 ml of ice cold collagenase solution (Worthington CL3, 200 units/ml in Earle's balanced salt solution). The cell suspension was drawn through an 18 ga. needle, filtered through cotton gauze, then drawn through a 25 ga. needle. This suspension was centrifuged at 833 X g for 5 min at 4°C. The supernatant was removed and replaced with 3 ml ice cold Earle's balanced salt solution. Fluorescence data (488 nm Ex, 526 nm Em) was collected with and without ethidium bromide (0.25 $\mu\text{g/ml}$) using a FACScan (Becton-Dickinson

Immunocytometry Systems, Mountain View, CA, 94039). Cell size (forward scatter) and cell granularity (side scatter) data were also collected.

HPLC was performed as previously described (Cox et al., 1990). Plasma samples (0.5 ml) were extracted using 1 c.c. Sep-Pak Vacs (Waters Assoc., Milford, MA, U.S.A.). Tumor samples (0.25 - 0.5 g) were homogenized in 2 ml of 0.05 M Na_2HPO_4 , pH 7.0 with daunomycin added as an internal standard. AgNO_3 (0.4 ml, 33% w/v) was added prior to homogenization to release Adriamycin bound to DNA (Schwartz, 1973). The homogenate was incubated for 10 minutes on ice. After centrifugation at 833 g for 5 minutes at 4°C the supernatant was treated like plasma.

CHAPTER 3: RESULTS

HSP 70

Tumors on anesthetized animals were heated (43.5°C for 1 hr), ³H-methionine was injected immediately at the end of heating, then rats were euthanized 3 hr later. ³H-methionine incorporation into a 70 kDa protein, thought to be HSP 70, was increased by 1 hyperthermia treatment ($p=0.004$) relative to unheated controls (Fig. 1). A second hyperthermia treatment 24 hr later also increased ³H-methionine incorporation relative to controls ($p=.007$), but not relative to 1 hyperthermia (Fig. 1). Longer ³H-methionine labelling did not increase incorporation (data not shown). 2 to 4 other proteins were seen in most samples from heated tumors, but these proteins exhibited little or no ³H-methionine incorporation.

Blood Flow

Tumor blood flow rates were determined using ¹¹³Sn microsphere injection. A slight, nonstatistically significant increase in tumor blood flow relative to unheated controls was seen immediately after the end of hyperthermia, but rates rapidly returned to near control levels (Fig. 2). Over the limited range of tumor volumes studied, no correlation between tumor volume and blood flow was seen (data not shown).

Adriamycin Determinations Using HPLC

ADR concentration of plasma samples was determined by HPLC and pharmacokinetic parameters were calculated. Data from the 30 min treatment group were fitted to a one compartment model, while data from the 60 min treatment group were fitted to a two compartment model. No significant difference between comparable treatment groups was seen (PROC TTEST, Statistical Analysis System (SAS), Cary NC, U.S.A.). The metabolites adriamycinol and adriamycin aglycone were seen near the detection limit in almost all immediate plasma samples; concentrations were not calculated. Six immediate (average time 5.5 min) plasma samples did contain measurable amounts of adriamycinol, although the area of the adriamycinol peak was always less than 5% of the ADR peak in a given sample. No pattern was seen regarding the occurrence of adriamycinol in any given treatment group.

ADR content of tumor samples was also determined using HPLC. Concentrations from hyperthermia treatment groups were compared with corresponding unheated groups (i.e. 30 min vs. 30 min) using PROC TTEST (SAS, Cary, NC, U.S.A.). Prior hyperthermia generally decreased the ADR content of tumors relative to unheated controls (0 HT, 30 min vs. 1 HT, 30 min, $p=0.008$; 0 HT, 60 min vs. 1 HT, 60 min, $p=.016$; 0 HT, 60 min vs. 2 HT, 60 min, $p=.007$) as shown in Fig. 3. An exception is 0 HT, 30 min vs. 2 HT, 30 min, where $p=0.104$. No metabolites were detected in tumor samples.

Adriamycin Determinations Using FACS

FACS was used to determine the ADR concentration of a small piece of each tumor. These data do not follow the trend seen in HPLC data. Instead, ADR content of tumor cells, as determined by FACS, is relatively unchanged by prior hyperthermia (Fig. 4).

CHAPTER 4: DISCUSSION

Other studies have documented *in vivo* HSP production in normal rat tissues after whole body hyperthermia (Fujio et al., 1987; Currie and White, 1983). Both studies reported a similar time course for HSP production, although shorter duration hyperthermia (15 min) induced maximum HSP production in both cases. While the present study did not attempt to unequivocally identify the 70 kDa protein as HSP 70, several studies have identified a 68-71 kDa protein produced in murine tumor cells after hyperthermia as HSP 70 (Fujio et al., 1987; Li and Mak, 1985; Landry et al., 1982; Tomasovic et al., 1983). The protein was isolated and characterized in some detail by Guidon and Hightower (1986) and Welch and Feramisco (1982).

Our finding of increases in blood flow to tumors at the end of hyperthermia is in agreement with that of Song et al. (1980). In that study radioactive microspheres were used to document a slight (17%) increase in blood flow to 0.3-0.7 gram Walker 256 transplanted tumors at the end of regional hyperthermia (43°C for 1 hr). Our blood flow data collected at later intervals conflict with reports of decreased blood flow, presumably due to vascular damage, in the hours following hyperthermia of transplanted tumors (Rappaport and Song, 1983; Song, 1980). Although Song (1984) reviews reports of vascular damage in transplanted tumors after 43-44°C hyperthermia, it is possible that such damage only occurs at higher temperatures in the DMBA-induced tumor model. Blood flow to unheated DMBA-induced rat mammary tumors has been reported as 0.025 ml/min per gram using ^{133}Xe clearance (Moller and Bojsen, 1975). As ^{133}Xe clearance has been reported to be subject to variability due to errors in injection and analysis of clearance curves (Jain and

Ward-Hartley, 1984), these differences are probably attributable to technique. Different anesthetic protocols may also play a role.

The differing results obtained for ADR concentration in tumors using HPLC compared to FACS are interesting. These differences can probably be explained by the fact that intracellular ADR is primarily bound to DNA; greater than 99% according to Gigli et al.(1988). Once bound to DNA, ADR fluorescence greatly reduced due to quenching (Egorin et al., 1980; Krishan and Ganapathi, 1979). The HPLC method used here utilized exposure of the tumor homogenate to AgNO_3 prior to analysis, causing the degradation of DNA and release of bound ADR (Schwartz, 1973). The FACS method, on the other hand, used whole, live cells and was capable of detecting primarily ADR that was not bound to DNA. One report of ADR detection using FACS after *in vitro* exposure utilized high, constant ADR exposure. Luk and Tannock (1984) observed constant intracellular ADR concentration only after 2 hr at 2 $\mu\text{g}/\text{ml}$. Speth et al. (1980) report detection of ADR in white cell subpopulations after 2 hr exposure to concentrations as low as 0.5 $\mu\text{g}/\text{ml}$. While these concentrations are attainable *in vivo*, they can be maintained only briefly after an intravenous bolus. Average plasma concentration in the present study immediately after drug administration (approx. 5 min) was 3.1 $\mu\text{g}/\text{ml}$ and was eliminated with a half-life of approximately 5 minutes. Krishan and Ganapathi (1979) report the use of an indirect propium iodide method for ADR detection.

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APPENDIX

FIGURES

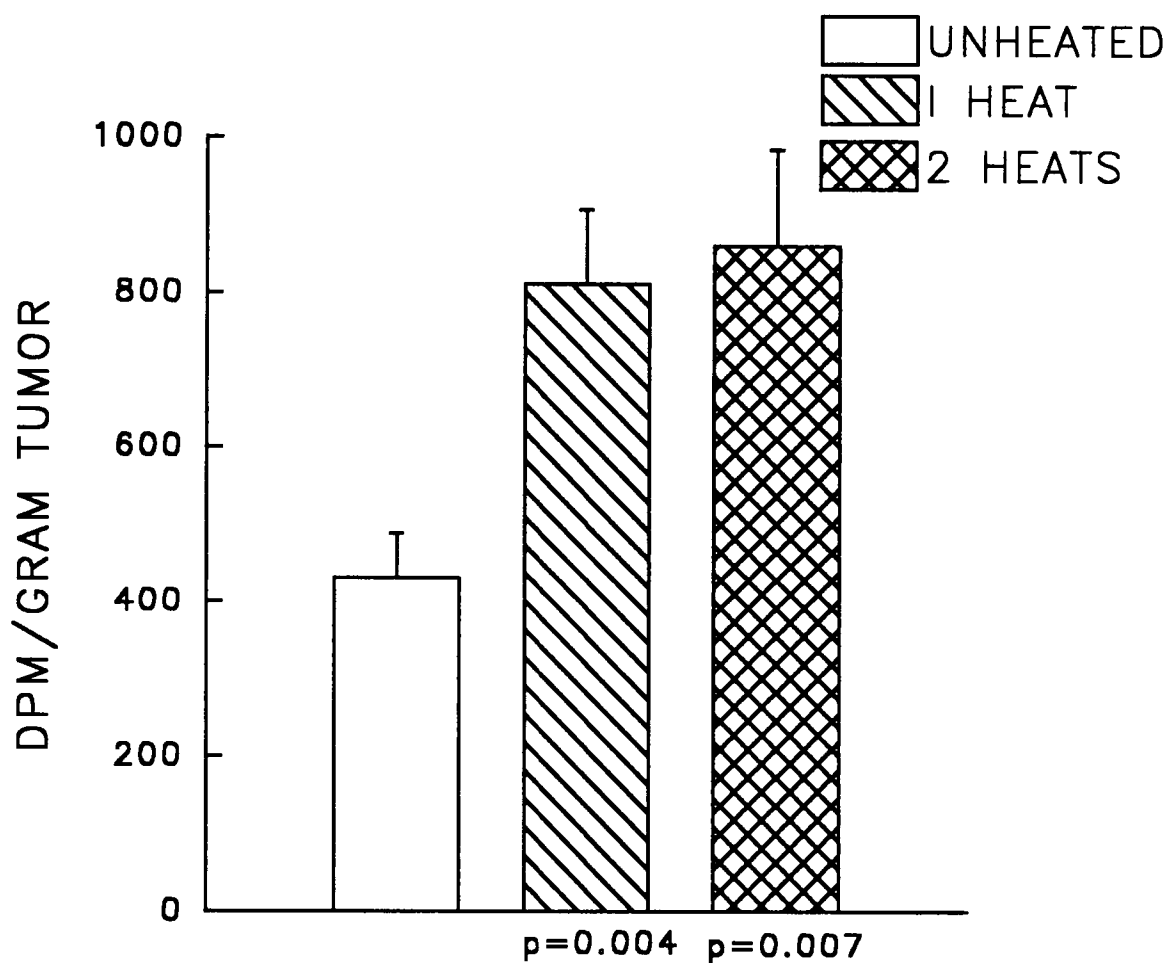


Figure 1. ^3H -methionine incorporation in HSP 70 from tumors heated 1 or 2 times (43.5°C for 1 hr). $200\ \mu\text{Ci}$ ^3H -methionine was injected i.v.; rats were euthanized 3 hr after injection. (mean $\pm$ SEM; $n=6$)

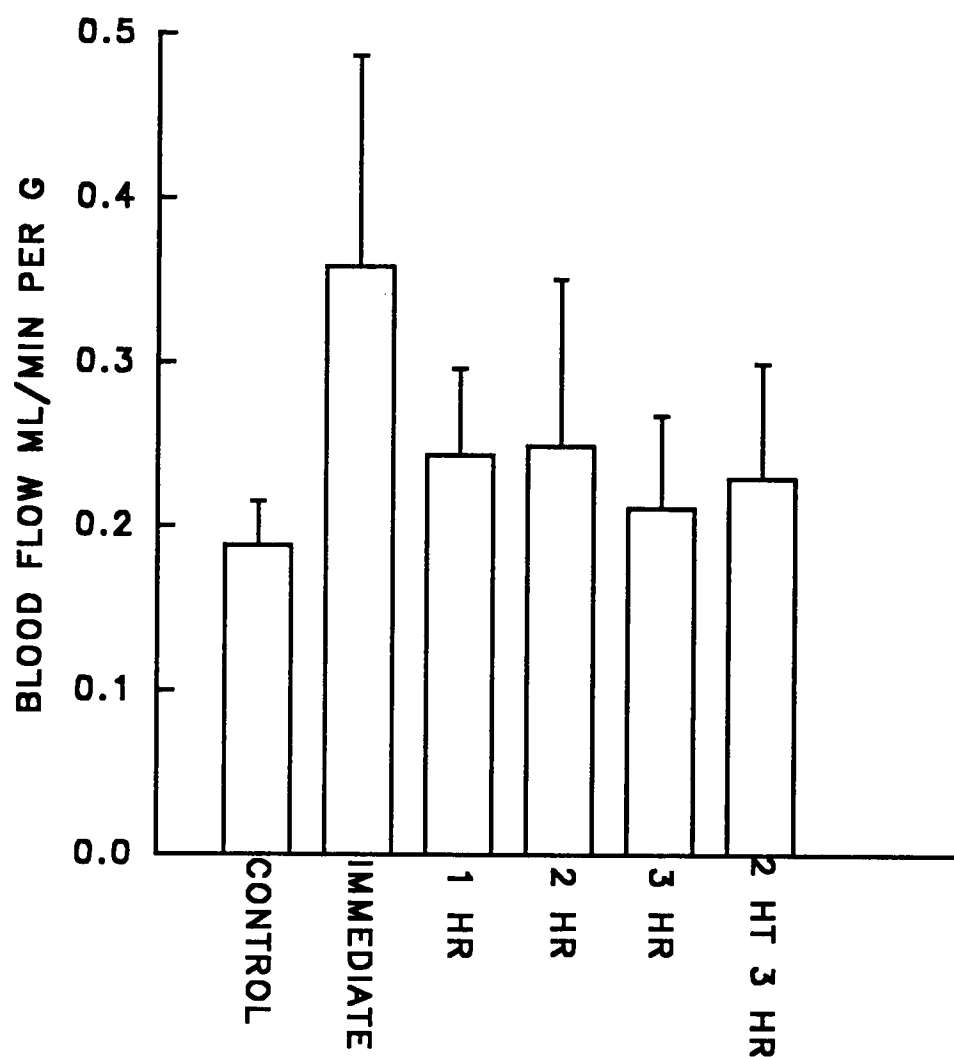


Figure 2. Blood flow rates to rat mammary tumors as determined by ^{113}Sn microsphere injection. Time after end of hyperthermia (43.5°C for 1 hr) is indicated. (mean $\pm$ SEM; $n=6$)

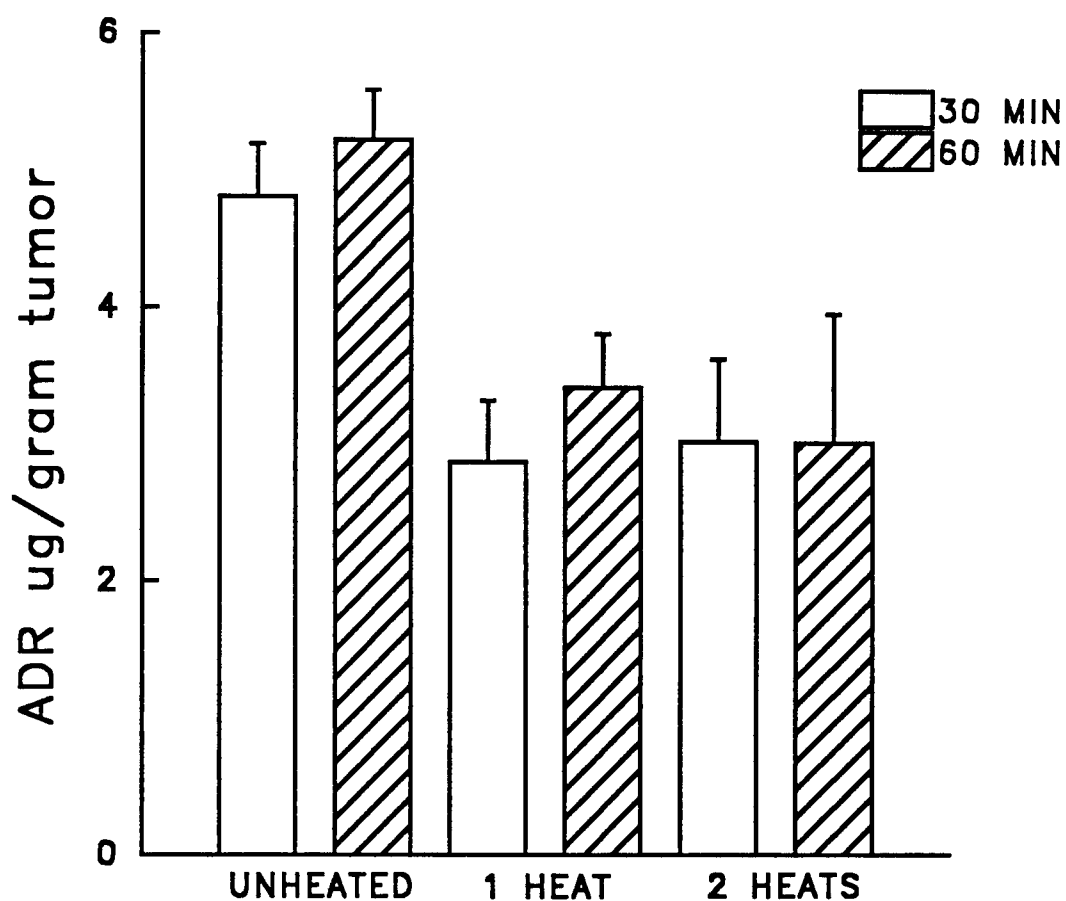


Figure 3. Adriamycin concentrations in rat mammary tumors 30 or 60 min after injection as determined by HPLC. Adriamycin (5 mg/kg, i.v.) was administered 3 hr after the end of 0, 1, or 2 hyperthermia treatments (43.5°C for 1 hr). (mean $\pm$ SEM; n=6)

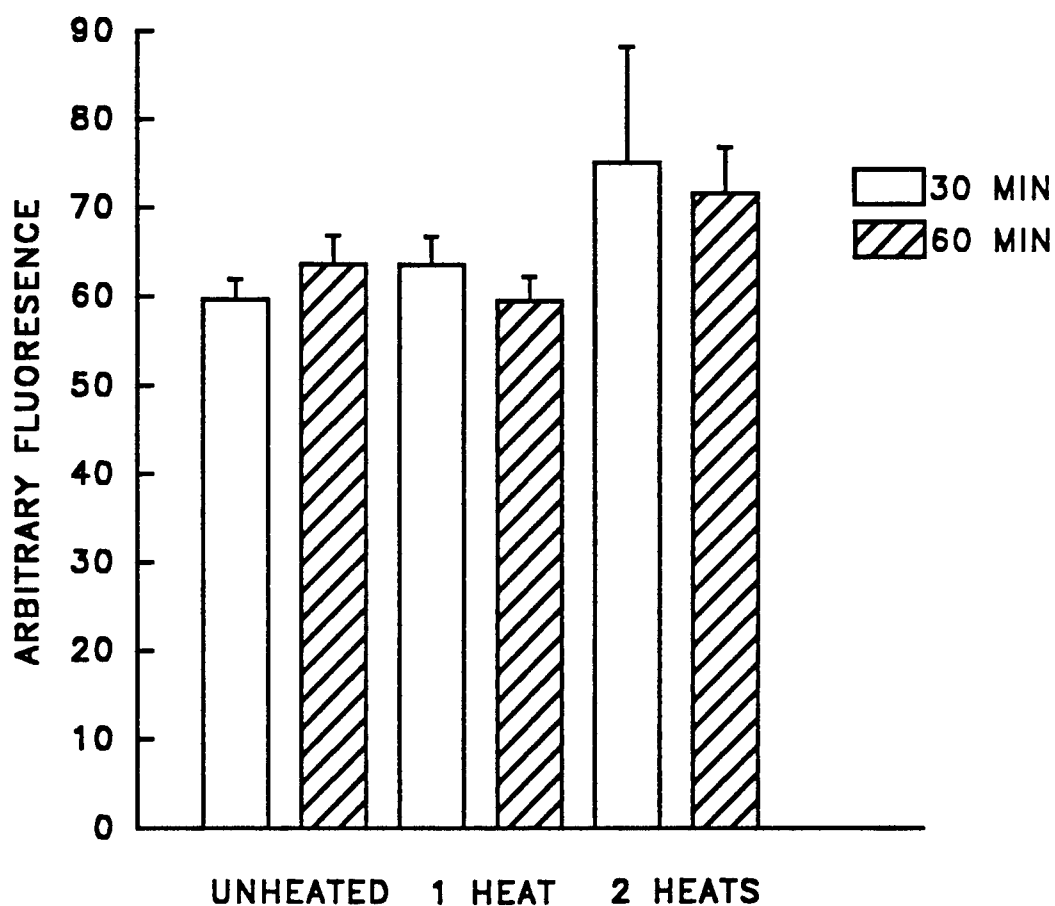


Figure 4. Adriamycin concentrations in rat mammary tumors 30 or 60 min after injection as determined by FACS. Adriamycin (5 mg/kg, i.v.) was administered 3 hr after the end of 0, 1, or 2 hyperthermia treatments (43.5°C for 1 hr). (mean $\pm$ SEM; n=6)

PART 4

RELATED STUDIES

CHAPTER 1: DETERMINATION OF ADRIAMYCIN IN PLASMA AND TISSUE BIOPSIES¹

Introduction

Adriamycin is an anthracycline antibiotic used in the treatment of a variety of malignant diseases including leukemias, lymphomas and carcinomas of the breast, lung, and ovary. Its clinical use is limited due to its cardiac toxicity (Andrews et al., 1980; Robert et al., 1982; Gessner et al., 1981). Numerous studies have been conducted in man and animals to determine adriamycin pharmacokinetics with different treatment modalities to try and minimize its toxicity while maintaining therapeutic dosages.

Adriamycin extraction from plasma using high performance liquid chromatography has been described by several groups (Robert, 1980; Cummings et al., 1984; Brenner et al., 1985; Bolanowska et al., 1983; Yazigi and Saleh, 1985; Israel et al., 1978; Van Lancker et al., 1986). These procedures involve the use of organic mixtures containing chloroform, hydrochloric acid - ethanol gels or C18 Sep Pak extractions with a vacuum pump. There are a few methods involving the extraction of adriamycin from tissues or cells (Van Lancker et al., 1986; Bolanowska and Gessner, 1982; Mimnaugh et al., 1978; Rahman et al., 1986; Cradock et al., 1973; Bachur et al., 1974; Cummings and McArdle, 1986; Ozols et al., 1979; Speth et al., 1986; Mahdadi et al., 1987). These methods use one gram of tissue and involve lengthy extractions with saturated solutions and

¹ This chapter has previously been published as "Determination of adriamycin in plasma and tissue biopsies", Cox SK, Wilke AV, and Frazier D, Journal of Chromatography, 564:322-329, 1991 and is used with the authors' permission.

organic mixtures, which are then analyzed either by thin layer chromatography, spectrofluorometry or high performance liquid chromatography.

This article describes a rapid and efficient C18 Sep Pak cleanup of plasma and small tissue samples and adriamycin analysis using high performance liquid chromatography. This procedure allows rapid analysis of tissue biopsies of tumors and normal tissue that may facilitate evaluation of the potential for toxicity and therapeutic efficiency of dosage regimens.

Experimental

Reagents and Standards

Acetonitrile and methanol were "HPLC" grade (Burdick & Jackson Laboratories, Inc., Muskegon, MI, USA). Ammonium formate, adriamycin and daunomycin, the internal standard, were purchased from Sigma Chemical Co., (St. Louis, MO, USA). NaH_2PO_4 , reagent grade, was obtained from Mallinckrodt, Inc., (Paris, KY, USA). The metabolite standards, adriamycinol, adriamycin aglycone, and adriamycinol aglycone, were gifts from Adria Laboratories, (Columbus, OH, USA).

Stock standard solutions of adriamycin and daunomycin were prepared by dissolving 5 mg each in 100 ml of HPLC grade methanol. Working standards of each were then made by serial dilutions of stock standards with methanol. Standards were stable at -20°C in brown bottles for one month. Metabolite standards were also prepared by dissolution in methanol.

Apparatus

The analytical system consisted of a model 600E solvent delivery system, a model 700 satellite WISP autosampler, a RCM 8 mm x 10 cm cartridge holder equipped with a 8 mm by 100 mm μ bondapak phenyl cartridge (10 μ m particle size) and a CN Guard-Pak precolumn insert, a model 470 scanning fluorescence detector and a NEC Powermate 2 computer system (Waters Associates, Milford, MA, USA).

Chromatography

The mobile phase consisted of acetonitrile and 0.1 M formate buffer. The formate buffer was prepared from ammonium formate and adjusted to pH 4 with 4 M hydrochloric acid. The solvent was used in a linear gradient from 73% A (ammonium formate), 27% B (acetonitrile) to 70% A, 30% B up to 12 min and back to 73% A, 27% B at 18 min. The flow rate was 2 ml/min and column temperature was ambient. Fluorescence detection of the peaks occurred with an excitation of 480 nm and an emission of 550 nm. All chromatograms were obtained with the following fluorometric conditions; gain 1000 x, attenuation 1 and filter time constant 0.5 sec.

Tissue Extraction Procedure

The tissue (5 to 100 mg) was placed in a Dounce glass tissue grinder (Baxter, McGraw Pk, Il, USA) and homogenized in 1.5 ml of 0.05 M NaH_2PO_4

solution (pH 7.0) with 10 to 15 strokes. One milliliter of the tissue/phosphate solution was transferred to a glass culture test tube and the internal standard daunomycin (25 μ l of a 5 μ g/ml solution) was added to the solution and mixed using a vortexer. This solution was added to a C18 Sep Pak (Waters Associates, Milford, MA, USA, Cat. no. 51910, used as a mini-chromatographic column) prewet with 3 ml methanol, 3 ml methanol:water (1:1) and 10 ml NaH_2PO_4 (0.05 M, pH 7.0). The tissue/phosphate solution was introduced onto the Sep Pak which was then washed with 3 ml of phosphate buffer and eluted with 3 ml methanol. The methanol solution was placed in a warm water bath (45°C) and evaporated to dryness under nitrogen. The residue was then reconstituted in 500 μ l of the mobile phase. The samples were briefly centrifuged (1000g for 5 min.) to remove any sediment and a 175 μ l aliquot of the clear supernatant was injected into the liquid chromatograph.

Plasma Extraction Procedure

Previously frozen plasma samples were thawed and mixed before use. Appropriate standard concentrations were evaporated under nitrogen to prevent methanol-induced protein precipitation upon addition of the plasma sample. The internal standard, daunomycin (25 μ l of a 5 μ g/ml solution), was added and mixed. The plasma samples were then extracted using the method described for tissue.

Results

A representative chromatogram for extracted tissue is shown in Figure 1. The retention times for adriamycin, adriamycinol, and daunomycin were 11.60, 7.26 and 19.44 minutes, respectively. The chromatogram in Figure 2 is a canine tissue sample collected 60 minutes after a 30 mg/m² dose of adriamycin. Retention times for adriamycin and daunomycin were 11.26 and 19.00 minutes. Endogenous peaks produced by the tissue did not appear after 2.25 minutes and, therefore, did not interfere with the elution of the anthracyclines. A representative chromatogram of the separation achieved in plasma is shown in Figure 3. Retention times for adriamycin, adriamycinol, adriamycin aglycone, adriamycinol aglycone, and daunomycin were 11.04, 6.79, 13.85, 8.60 and 18.33 minutes, respectively. Figure 4 is a chromatogram of canine plasma collected 45 minutes after administration of 30 mg/m² of adriamycin. Adriamycin, adriamycinol and daunomycin retention times were 10.64, 6.05 and 19.01 minutes, respectively. Similar to tissue, peaks resulting from endogenous plasma components occurred prior to 2.62 minutes.

The method for tissue analysis produced a linear curve for the concentration range used in this study, with the correlation coefficients ranging from 0.998 to 0.999. Replicate analyses performed on the same day for tissues spiked with specific concentrations of anthracyclines showed coefficients of variation to be 7.1% for 30 ng/mg, 6.2% for 75 ng/mg and 7.6% for 150 ng/mg (Table 1). Day-to-day variability for tissue replications are shown in Table 2. The mean percent recoveries of adriamycin from tissue were 81%, 85%, 81%, 87% and 80% for 10, 25, 50, 100 and 250 ng/mg, respectively. Adriamycin

detection limit in tissue was 2 ng/mg. This would represent a peak approximately 3 times baseline noise.

Correlation coefficients for the linear plasma curve ranged from 0.995 to 0.999. Results of between day replications for plasma are shown in Table 3. The mean percent recoveries for adriamycin in plasma were 80%, 82%, 89%, 73%, 70%, 75%, and 90% for 25, 50, 100, 250, 500, 800 and 1000 ng/ml, respectively. The detection limit for adriamycin in plasma was 5 ng/ml. This represented a peak approximately 3 times baseline noise at the highest sensitivity on the fluorescence detector.

DISCUSSION

Pharmacokinetic and pharmacodynamic studies of adriamycin and its metabolites require analysis of both tissue and plasma. It is often impossible to remove large amounts of tissue from cancer patients or experimental animals, therefore, an accurate reproducible procedure for small tissue biopsies is often needed. The procedure developed here has allowed the analysis of adriamycin tissue biopsies weighing less than 100 mg.

The detection limits and recoveries for both plasma and tissue samples are of equal sensitivity to existing methods for extraction and analysis of adriamycin. Reported plasma and tissue adriamycin concentrations in human patients and experimental animals range between 5 ng/ml and 1000 ng/ml. This method should be appropriate for patients and experimental animals administered therapeutic dosages of adriamycin.

The chromatograms do confirm the presence of an early peak due to endogenous plasma and tissue components. This did not interfere with the elution of adriamycin or its metabolites. There are different retention times for plasma and tissue samples. Theoretically they should be the same, but the different sample matrices caused a slight change in retention times.

The assay distinguished well between adriamycin and its most common metabolites, adriamycinol, adriamycinol aglycone, and adriamycin aglycone all of which have been identified in humans and animals (Brenner et al., 1985; Mimnaugh et al., 1978; Bachur et al., 1974; Cummings and McArdle, 1986). This procedure has been used for analysis of canine samples collected from veterinary patients and experimental animals in our institution. Metabolites were detected only sporadically in the dog and have been described quantitatively in a paper by

Wilke et al. (1991). The use of daunomycin as an internal standard allows compensation for intra- and inter-assay variability in the extraction and chromatography steps.

Other HPLC procedures using similar extractions for plasma such as Robert (1980) or Van Lancker et al. (1986), use either a chloroform-methanol or chloroform-isopropanol mixture for eluting the anthracyclines, while Yazigi and Saleh (1985) attach a vacuum pump to the C18 Sep Paks before eluting the samples. The use of a vacuum pump for large numbers of samples is time consuming and its lack of use did not appear to affect our results. Methanol was found to work as well as or better than the chloroform-methanol or chloroform-isopropanol mixtures in the elution of the anthracyclines. This change eliminated the use of chloroform in the procedure. The extraction procedure for tissue used by Bolanowska and Gessner (1982) used whole tissues and a chloroform-isopropanol (1:1, v/v) solution which was mixed with saturated sodium bicarbonate and sodium chloride. Bachur et al. (1974), Mimnaugh et al. (1978) and Cradock et al. (1973) used one gram of tissue and a chloroform-methanol (2:1, v/v) mixture in their extraction procedures. Anthracyclines were extracted from cell culture medium by Van Lancker et al. (1986) using an ice-cold chloroform-isopropanol (4:1, v/v) mixture. Speth et al. (1986) and Mahdadi et al. (1987) extracted anthracyclines from human haematopoietic cells and rat hepatocytes using either a chloroform-methanol (9:1, v/v) or chloroform-butanol solution (1:1, v/v). Our procedure eliminated the use of organic mixtures and was accurate for 5-100 mg tissue samples.

In conclusion, a rapid, sensitive and clinically useful HPLC procedure has been developed for analysis of adriamycin in plasma and small tissue biopsies. This method would be appropriate for analyzing large numbers of samples.

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Table 1. Within assay precision for spiked tissue standards. Spiked concentration (Concentration, ng Adriamycin/5 mg tissue) and the mean of experimentally determined concentrations (Mean) are shown. Standard deviation (S.D.) and coefficient of variation (C.V.), are also shown.

Concentration	N	Mean	S.D.	C.V. (%)
30	4	28.0	2.5	7.1
75	4	72.2	5.2	6.2
150	4	152.0	12.4	7.6

Table 2. Between-day assay precision for spiked tissue standards. Spiked concentration (Concentration, ng Adriamycin/5 mg tissue), experimentally determined concentration (Measured Concentration), and the mean ratio of Adriamycin to daunomycin (Mean Area Ratio) are shown. Standard deviation (S.D.) and coefficient of variation (C.V.), are also shown.

Concentration	N	Measured Concentration	Mean Area Ratio	S.D.	C.V. (%)
10	4	8	0.02829	0.0013	3.5
25	4	22	0.11232	0.0016	2.0
50	4	43	0.21532	0.0190	8.8
100	4	87	0.42355	0.0202	4.7
250	4	148	1.09930	0.0502	4.5

Table 3. Between-day assay precision for spiked plasma standards. Spiked concentration (Concentration, ng Adriamycin/ml plasma), experimentally determined concentration (Measured Concentration), and the mean ratio of Adriamycin to daunomycin (Mean Area Ratio) are shown. Standard deviation (S.D.) and coefficient of variation (C.V.), are also shown.

Concentration	N	Measured Concentration	Mean Area Ratio	S.D.	C.V. (%)
25	4	20	0.24609	0.011	4.5
50	4	41	0.35333	0.031	8.8
100	4	80	0.71579	0.053	7.4
250	4	182	1.81598	0.058	3.2
500	4	365	3.64940	0.254	7.0
800	4	586	9.22701	0.524	5.7
1000	4	925	10.84006	0.412	3.8

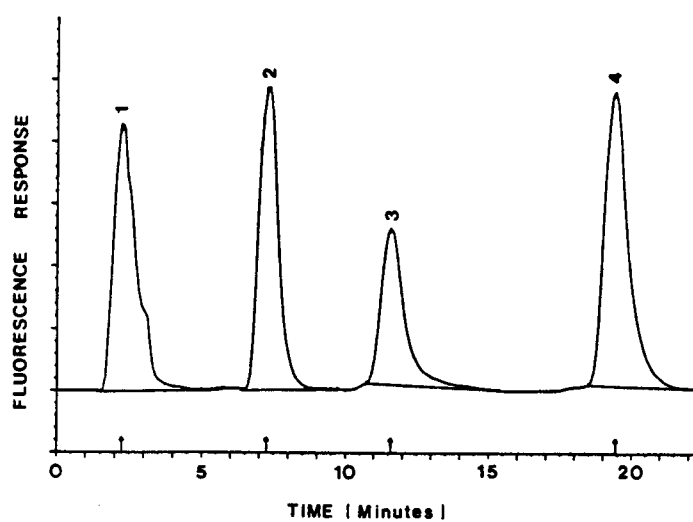


Figure 1. Chromatogram of a mixture of adriamycinol (2), adriamycin (3), and daunomycin (4) in tissue (100 ng/5 mg spiked tissue). Peak one results from endogenous tissue components.

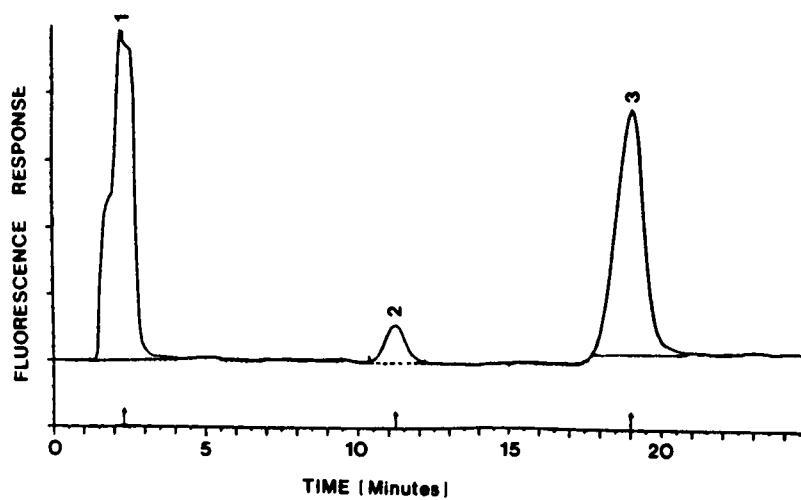


Figure 2. Chromatogram of a tissue sample collected 60 minutes after administration of 30 mg/m^2 adriamycin to a canine. Endogenous tissue components (1), adriamycin (2), and daunomycin (3) were detected.

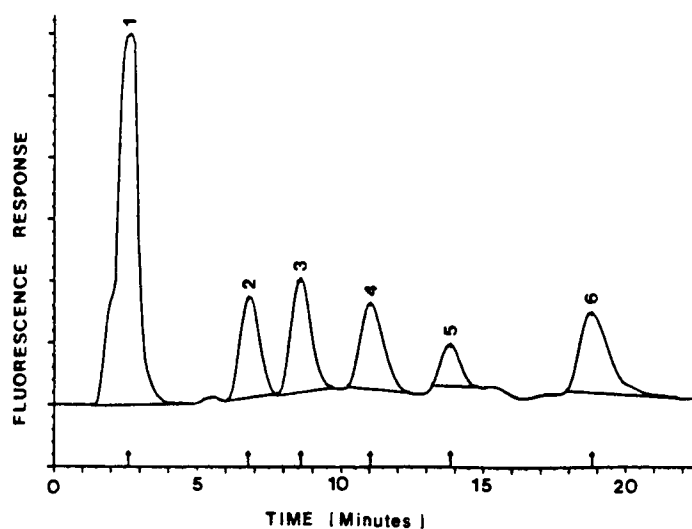


Figure 3. Chromatogram of a mixture of adriamycinol (2), adriamycinol aglycone (3), adriamycin (4), adriamycin aglycone (5), and daunomycin (6) (100 ng/ml spiked plasma). Peak one results from endogenous plasma components.

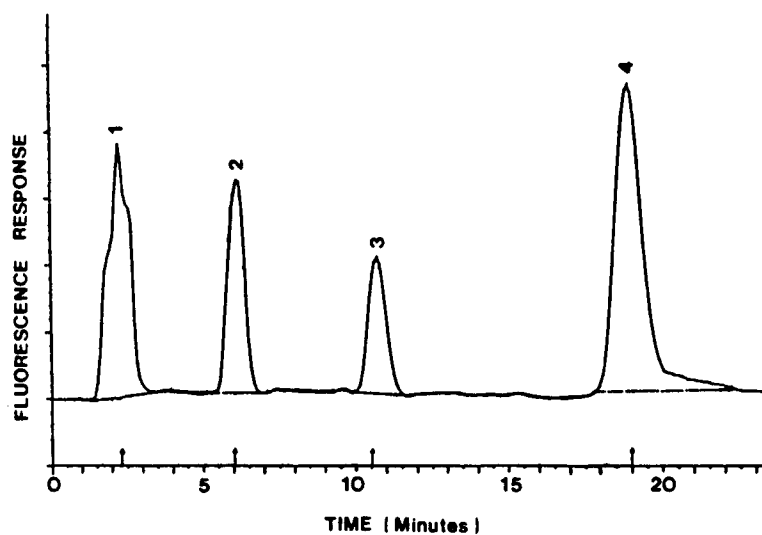


Figure 4. Chromatogram of a plasma sample collected 15 minutes after administration of 30 mg/m² adriamycin to a canine. Endogenous plasma components (1), adriamycinol (2), adriamycin (3), and daunomycin (4) were detected.

CHAPTER 2: ND:YAG LASER HYPERTHERMIA OF A RAT MAMMARY ADENOCARCINOMA WITH ACCOMPANYING SURFACE COOLING¹

Rat Tumor Model

50 day old female Sprague-Dawley rats were administered 9,10-dimethyl-1,2-benzanthracene (DMBA) (10 mg/100 g) by gavage while under halothane anesthesia. DMBA was dissolved in sesame oil (10 mg/ml). Mammary adenocarcinomas appeared 5-10 weeks later. Tumors were treated when 1 cm in diameter. Rats were anesthetized prior to treatment (0.125 mg acepromazine maleate and 6.25 mg ketamine HCl, i.m.).

Hyperthermia

Hyperthermia (43.5°C, 1 hr) was induced using a Nd:YAG laser (Surgical Laser Technologies, CL60, Tokyo, Japan) which emits light in the near infrared region (1064 nm). This wavelength is minimally absorbed by water, hemoglobin, and melanin allowing good tissue penetration. The laser was controlled by a K-type feedback probe placed near the base of the tumor. Three T-type probes in 24 ga. catheters were placed just under the skin, at the tumor center, and at the tumor base. Data was used only when the average temperature at the center of the probe was 43.5°C $\pm$ 5%. Average temperatures for each rat over the course of 1 hr at the three locations are shown in Table 4.

¹ A significant portion of this chapter will be published as "Nd:YAG laser hyperthermia treatment of rat mammary adenocarcinoma in conjunction with surface cooling", Panjehpour M, Wilke AV, Frazier DL, and Overholt BF, *Lasers in Surgery and Medicine*, 1991 (in press) and is used with the authors' permission.

Although penetration of tissue by the Nd:YAG light was good, surface cooling was necessary in order to adequately heat the tumor base without overheating the surface. Wall oxygen directed through a water nebulizer normally used in respiratory therapy and set at 100% humidity was used. When directed at the tumor surface, this allowed power to be increased, heating the tumor center and base without overheating the surface. This phenomenon is demonstrated in Figures 5-7. When cooling was used, power in the range of 8-12 watts was used.

Table 4. Mean tumor temperatures ($^{\circ}\text{C}$) over 1 hour obtained using Nd:YAG laser hyperthermia with concurrent surface cooling. Number of hyperthermia treatments (1 or 2) and treatment arm (ADR = Adriamycin; HSP = HSP 70; BF = Blood Flow) are also indicated.

Heats, Arm	Rat	Surface Temp $\pm$ Std error	Center Temp $\pm$ Std error	Base Temp $\pm$ Std error
1, ADR	526	44.7 $\pm$ 0.2	45.0 $\pm$ 0.2	42.2 $\pm$ 0.1
	532	42.9 $\pm$ 0.2	42.7 $\pm$ 0.1	41.8 $\pm$ 0.1
	538	44.3 $\pm$ 0.1	43.9 $\pm$ 0.2	40.0 $\pm$ 0.1
	554	44.1 $\pm$ 0.1	44.6 $\pm$ 0.1	38.2 $\pm$ 0.1
	610	41.4 $\pm$ 0.2	43.4 $\pm$ 0.1	38.9 $\pm$ 0.1
	617	42.4 $\pm$ 0.2	44.7 $\pm$ 0.1	42.9 $\pm$ 0.1
	647	48.0 $\pm$ 0.2	48.0 $\pm$ 0.2	40.7 $\pm$ 0.1
	483	42.9 $\pm$ 0.1	44.1 $\pm$ 0.1	41.2 $\pm$ 0.1
	612	44.4 $\pm$ 0.1	43.7 $\pm$ 0.1	40.2 $\pm$ 0.1
	625	44.2 $\pm$ 0.1	43.5 $\pm$ 0.1	39.1 $\pm$ 0.1
	658	44.6 $\pm$ 0.2	44.6 $\pm$ 0.1	41.5 $\pm$ 0.1
	661	42.6 $\pm$ 0.1	44.0 $\pm$ 0.1	43.9 $\pm$ 0.1
	698	43.2 $\pm$ 0.1	44.0 $\pm$ 0.1	42.6 $\pm$ 0.1
2, ADR	485	41.4 $\pm$ 0.1	43.3 $\pm$ 0.1	43.1 $\pm$ 0.1
	485b	42.8 $\pm$ 0.1	43.7 $\pm$ 0.1	43.2 $\pm$ 0.1
	490	39.5 $\pm$ 0.1	41.8 $\pm$ 0.1	41.6 $\pm$ 0.1
	490b	41.1 $\pm$ 0.2	45.6 $\pm$ 0.2	44.7 $\pm$ 0.2
	501	40.5 $\pm$ 0.3	42.3 $\pm$ 0.1	42.4 $\pm$ 0.1
	501b	43.3 $\pm$ 0.3	44.4 $\pm$ 0.2	41.6 $\pm$ 0.1
	505	43.0 $\pm$ 0.2	42.9 $\pm$ 0.2	43.2 $\pm$ 0.1
	505b	42.5 $\pm$ 0.1	44.6 $\pm$ 0.1	41.0 $\pm$ 0.1
	599	42.8 $\pm$ 0.1	44.0 $\pm$ 0.1	42.5 $\pm$ 0.1
	599b	44.0 $\pm$ 0.1	45.4 $\pm$ 0.1	42.1 $\pm$ 0.1
	639	45.4 $\pm$ 0.2	43.7 $\pm$ 0.1	41.0 $\pm$ 0.1
	639b	44.3 $\pm$ 0.1	43.2 $\pm$ 0.1	41.6 $\pm$ 0.1
	519	38.0 $\pm$ 0.2	43.9 $\pm$ 0.2	43.1 $\pm$ 0.1
	519b	44.2 $\pm$ 0.2	43.5 $\pm$ 0.2	43.3 $\pm$ 0.1
	567	44.4 $\pm$ 0.1	44.6 $\pm$ 0.1	41.9 $\pm$ 0.1
	567b	42.4 $\pm$ 0.1	43.8 $\pm$ 0.1	42.1 $\pm$ 0.1
	569	43.5 $\pm$ 0.1	43.7 $\pm$ 0.1	39.3 $\pm$ 0.0
	569b	43.3 $\pm$ 0.1	43.5 $\pm$ 0.1	39.5 $\pm$ 0.0
	572	42.0 $\pm$ 0.1	43.6 $\pm$ 0.1	42.1 $\pm$ 0.1
	572b	43.9 $\pm$ 0.2	44.3 $\pm$ 0.1	43.3 $\pm$ 0.1
1, HSP	675	43.9 $\pm$ 0.2	43.3 $\pm$ 0.1	42.7 $\pm$ 0.1
	675b	44.6 $\pm$ 0.2	43.9 $\pm$ 0.1	41.0 $\pm$ 0.1
	688	42.0 $\pm$ 0.1	43.6 $\pm$ 0.2	41.6 $\pm$ 0.1
	688b	44.4 $\pm$ 0.1	44.8 $\pm$ 0.1	42.1 $\pm$ 0.1
	689	44.8 $\pm$ 0.2	43.9 $\pm$ 0.1	39.0 $\pm$ 0.0
	689b	44.1 $\pm$ 0.2	42.5 $\pm$ 0.1	38.4 $\pm$ 0.1
	472	44.3 $\pm$ 0.2	45.2 $\pm$ 0.2	40.0 $\pm$ 0.1
	484	40.5 $\pm$ 0.1	41.9 $\pm$ 0.1	42.4 $\pm$ 0.1
	492	41.0 $\pm$ 0.4	43.0 $\pm$ 0.4	40.0 $\pm$ 0.2
	496	34.1 $\pm$ 0.1	40.6 $\pm$ 0.1	40.8 $\pm$ 0.1
	498	44.6 $\pm$ 0.2	42.4 $\pm$ 0.2	41.3 $\pm$ 0.1

Heats, Arm	Rat	Surface Temp $\pm$ Std Err	Center Temp $\pm$ Std Err	Base Temp $\pm$ Std Err
2, HSP	506	40.9 $\pm$ 0.1	42.9 $\pm$ 0.1	44.0 $\pm$ 0.1
	598	44.6 $\pm$ 0.2	44.2 $\pm$ 0.1	42.6 $\pm$ 0.1
	445	34.0 $\pm$ 0.2	40.3 $\pm$ 0.1	43.3 $\pm$ 0.1
	445b	43.4 $\pm$ 0.2	43.2 $\pm$ 0.1	43.5 $\pm$ 0.1
	450	46.2 $\pm$ 0.2	46.4 $\pm$ 0.2	41.2 $\pm$ 0.1
	450b	41.7 $\pm$ 0.3	41.0 $\pm$ 0.4	37.4 $\pm$ 0.2
	493	42.6 $\pm$ 0.1	45.4 $\pm$ 0.2	39.9 $\pm$ 0.1
	493b	44.8 $\pm$ 0.2	42.5 $\pm$ 0.2	41.4 $\pm$ 0.1
	515	40.1 $\pm$ 0.2	43.2 $\pm$ 0.1	42.9 $\pm$ 0.1
	515b	39.4 $\pm$ 0.3	40.8 $\pm$ 0.3	42.3 $\pm$ 0.2
	652	42.7 $\pm$ 0.1	44.2 $\pm$ 0.1	42.2 $\pm$ 0.1
	652b	43.0 $\pm$ 0.1	44.3 $\pm$ 0.1	42.5 $\pm$ 0.1
	695	44.9 $\pm$ 0.1	45.4 $\pm$ 0.1	42.5 $\pm$ 0.1
	695b	44.8 $\pm$ 0.2	44.1 $\pm$ 0.1	40.0 $\pm$ 0.1
1, BF	287	37.9 $\pm$ 0.2	43.5 $\pm$ 0.5	41.6 $\pm$ 0.1
	408	37.4 $\pm$ 0.2	43.5 $\pm$ 0.2	41.9 $\pm$ 0.2
	470	31.0 $\pm$ 0.5	42.4 $\pm$ 0.3	42.4 $\pm$ 0.3
	531	43.8 $\pm$ 0.1	43.9 $\pm$ 0.1	43.1 $\pm$ 0.1
	535	44.8 $\pm$ 0.1	44.0 $\pm$ 0.2	43.4 $\pm$ 0.1
	584	43.1 $\pm$ 0.2	43.5 $\pm$ 0.2	43.8 $\pm$ 0.2
	649	42.8 $\pm$ 0.1	43.5 $\pm$ 0.1	44.0 $\pm$ 0.1
	650	43.5 $\pm$ 0.1	43.5 $\pm$ 0.2	43.2 $\pm$ 0.2
2, BF	8	44.0 $\pm$ 0.1	43.4 $\pm$ 0.1	43.4 $\pm$ 0.1
	8b	44.0 $\pm$ 0.1	43.3 $\pm$ 0.1	41.5 $\pm$ 0.1
	10	44.3 $\pm$ 0.1	44.0 $\pm$ 0.1	43.0 $\pm$ 0.1
	10b	44.5 $\pm$ 0.1	44.0 $\pm$ 0.1	42.5 $\pm$ 0.1
	607	43.9 $\pm$ 0.2	44.7 $\pm$ 0.2	41.3 $\pm$ 0.1
	677	43.9 $\pm$ 0.1	44.4 $\pm$ 0.1	42.8 $\pm$ 0.1
	677b	42.9 $\pm$ 0.1	43.9 $\pm$ 0.1	41.9 $\pm$ 0.1
	691	43.9 $\pm$ 0.1	43.9 $\pm$ 0.1	42.7 $\pm$ 0.1
	691b	43.0 $\pm$ 0.1	43.5 $\pm$ 0.1	40.7 $\pm$ 0.1

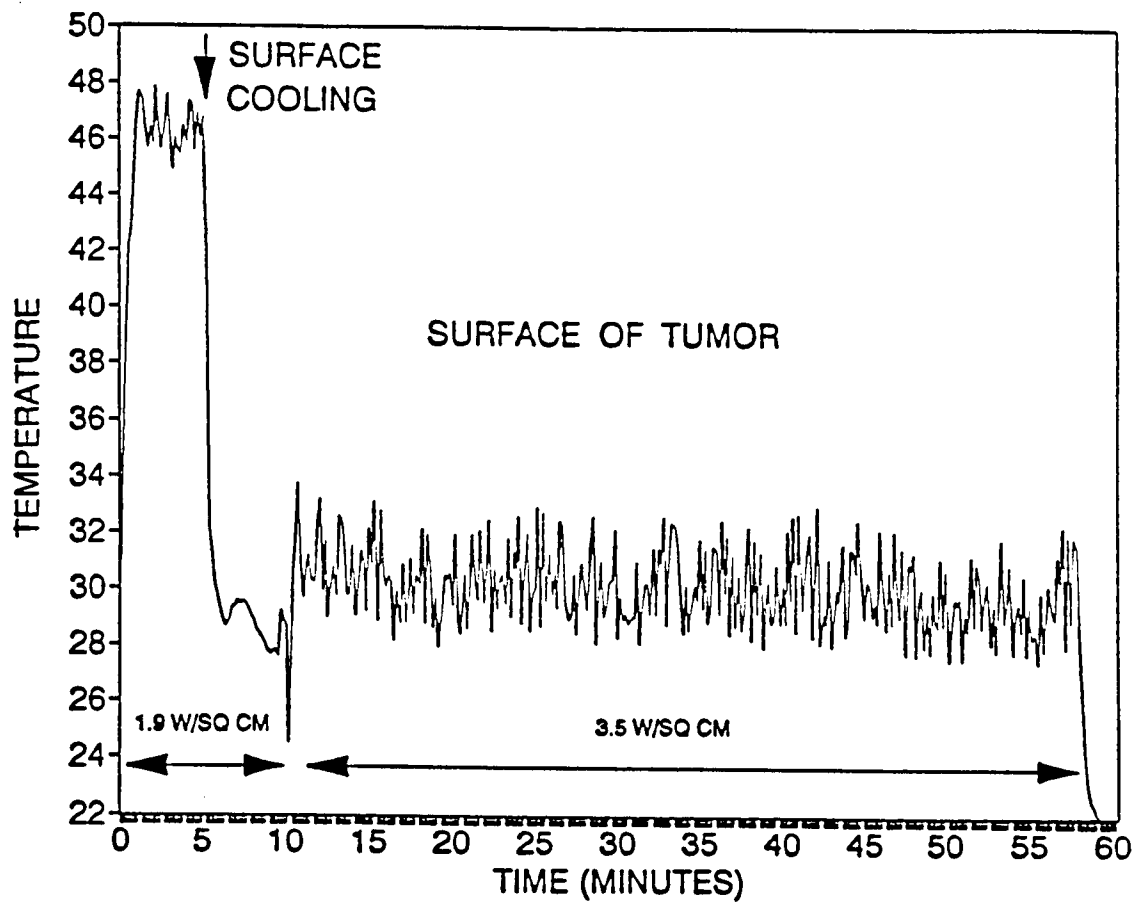


Figure 5. The effect of laser hyperthermia with and without surface cooling on temperature just under the skin in a rat mammary adenocarcinoma. Laser hyperthermia was used initially without surface cooling (time = 0 minutes), then surface cooling was added at 5 minutes and continued for the remainder of the treatment.

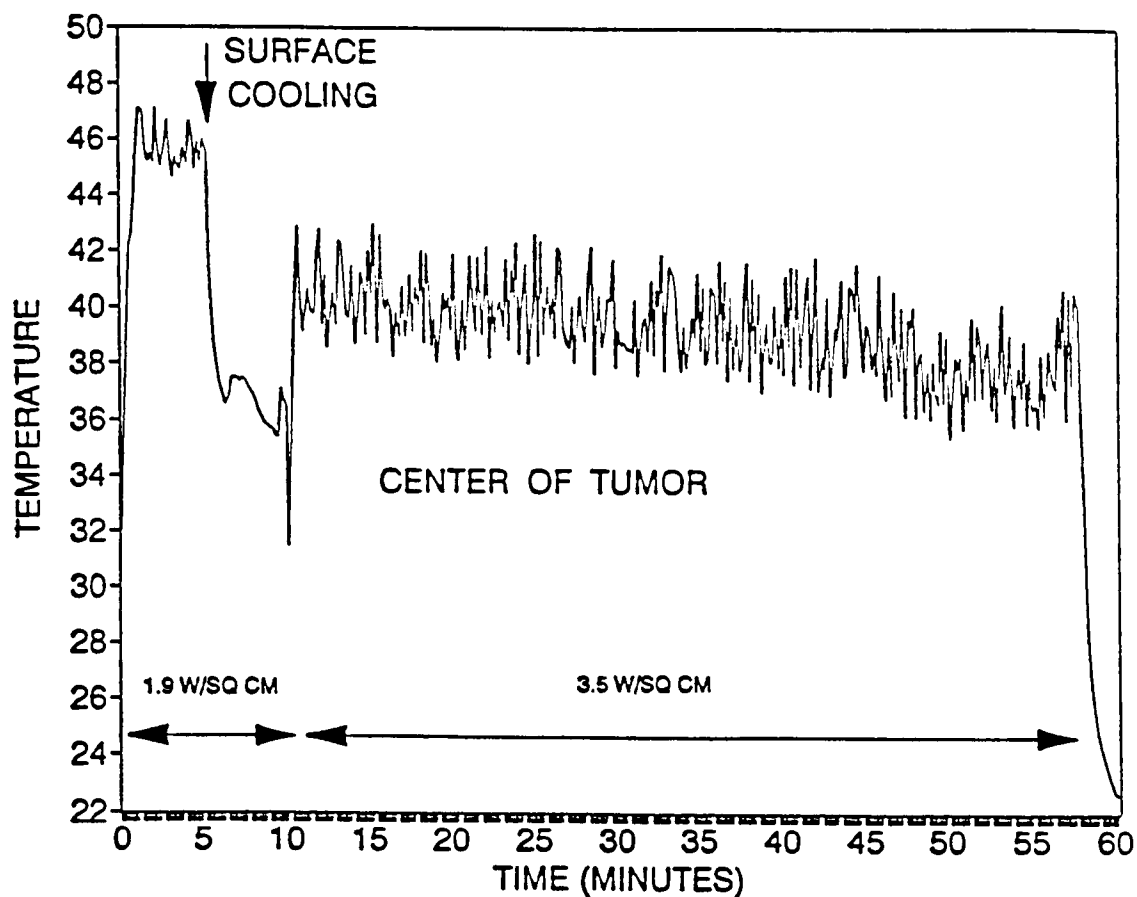


Figure 6. The effect of laser hyperthermia with and without surface cooling on temperature in the center of a rat mammary adenocarcinoma. Laser hyperthermia was used initially without surface cooling (time=0 minutes), then surface cooling was added at 5 minutes and continued for the remainder of the treatment.

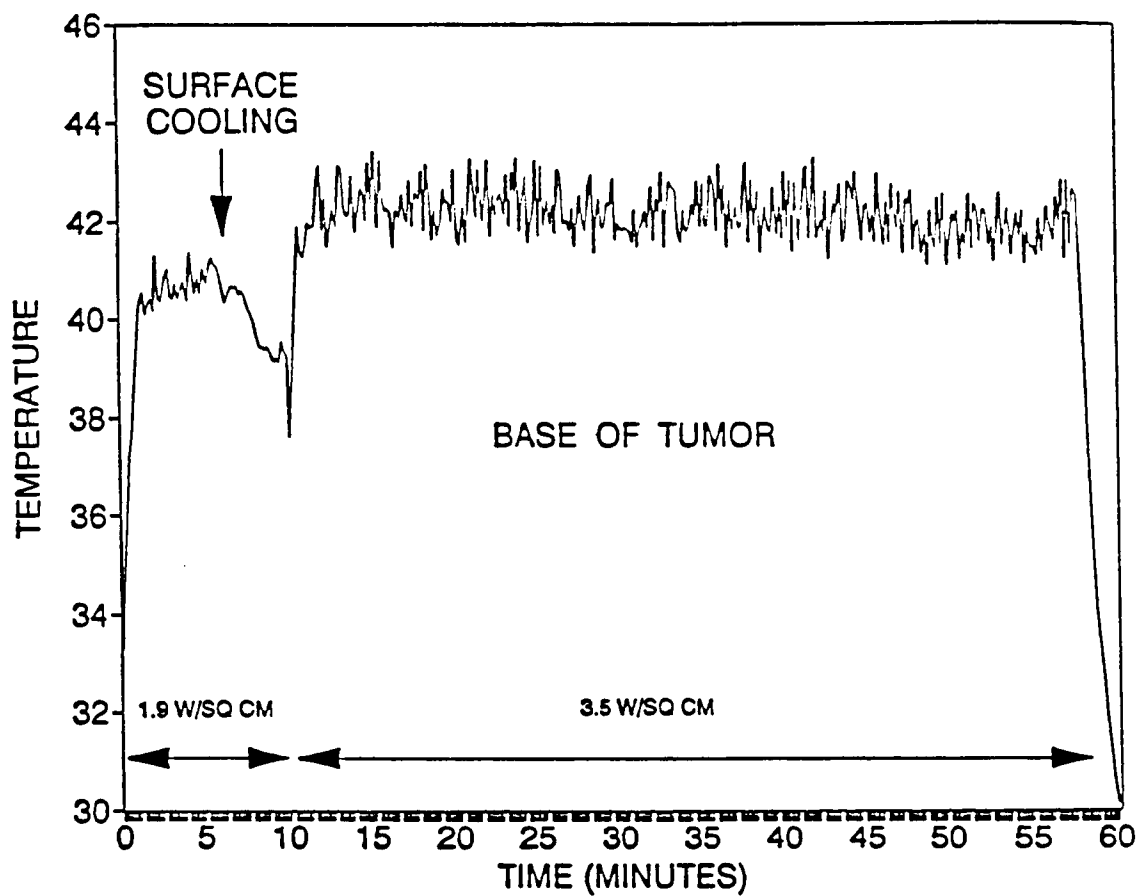


Figure 7. The effect of laser hyperthermia with and without surface cooling on temperature at the base of a rat mammary adenocarcinoma. Laser hyperthermia was used initially without surface cooling (time = 0 minutes), then surface cooling was added at 5 minutes and continued for the remainder of the treatment.

PART 5

SUMMARY

CHAPTER 1: CONCLUSIONS

In summary, hyperthermia has significant effects on the pharmacokinetics of Adriamycin in both plasma and tissues. Mild whole-body hyperthermia (42°C) in the normal dog has been shown to speed the distribution of Adriamycin from plasma to the tissues and increase the volume of distribution of Adriamycin. Whether whole-body hyperthermia could be used to increase Adriamycin delivery to a solid tumor was not examined, although such an effect seems likely. The effect of whole-body hyperthermia on tumor concentration of Adriamycin would likely depend on the location of the tumor as the effect of whole-body hyperthermia on blood flow varies between tissues. Drug delivery to the duodenum was examined and found to be increased, although no overt toxicity to the duodenum was seen. Using ^3H -thymidine, cell turnover in the duodenum reached maximum depression on Day 3 following Adriamycin, whether whole-body hyperthermia was administered or not.

Local hyperthermia (43.5°C) was shown to decrease Adriamycin concentrations in rat mammary tumors when it proceeded drug administration by 3 hr. This effect was not caused by reduced blood flow to tumors. Blood flow was slightly increased at the end of hyperthermia, but then returned to near control levels by 1 hr. The production of HSP 70 in tumor tissue *in vivo* was demonstrated. HSP 70 production was induced by 1 or 2 hyperthermia treatments; no significant difference in the amount of HSP 70 produced after 1 or 2 treatments was seen. The correlation between HSP 70 and reduced Adriamycin concentration after hyperthermia implies, but certainly does not prove, a causal relationship. A relationship between thermotolerance (or HSP expression) and Adriamycin resistance has been documented *in vitro*.

In conjunction with these studies, a method for quantifying Adriamycin and its major metabolites in plasma, small tissue biopsies, and tumor samples using HPLC with fluorescence detection was developed. A fluorescence-activated cell sorter (FACS) technique was also developed, although this technique failed to demonstrate any difference in intracellular Adriamycin concentrations between treatment groups.

A new method for inducing local hyperthermia in superficial tumors was also developed. This involved the use of a Nd:YAG laser with microcomputer feedback control and concurrent surface cooling of the tumor. It was demonstrated that this method could reproducibly provide uniform hyperthermia (43.5°C) in tumors up to at least 1 cm³.

CHAPTER 2: FUTURE DIRECTIONS

The study outlined above raises several questions that I would like to pursue. The first concerns the timing of administration of any antineoplastic agent relative to hyperthermia. There seem to be definite pharmacokinetic and pharmacodynamic differences that accompany the use of local or whole-body hyperthermia, and that vary with temperature. If slower heating of tissues increases resistance to Adriamycin, as some studies report, should Adriamycin be used with whole-body hyperthermia where heating the patient routinely takes 60-90 minutes? With respect to local hyperthermia, if drug delivery is reduced after hyperthermia, would it be efficacious to administer drug prior to hyperthermia, then use hyperthermia to trap drug in tumors?

The area of heat-shock response is also intriguing. Although an understanding of the heat-shock response as a phenomenon is emerging, some basic questions are still unanswered. How does a cell sense an increase in temperature? Is the induction of this response by certain chemicals an artifact and, if not, what is the common mechanism? How can the production of heat-shock proteins protect the cell from heat or certain chemicals?

VITA

August Wilke, III was born December 7, 1964 in Montgomery, Alabama. He graduated from Carver High School there in 1983, then attended Huntingdon College in Montgomery on an academic scholarship. As a Pre-Medicine major he was funneled into the chemistry program, but simultaneously became less interested in medical school and more interested in biology. He continued to take more biology courses, but continued the chemistry curriculum as well, eventually graduating with a double major. He came to the University of Tennessee to pursue a Ph.D. in Environmental Toxicology, but again had a shift in interest, becoming involved in oncology research.