ROLE OF GLUTATHIONE S-TRANSFERASES IN THE RESISTANCE OF HUMAN COLON CANCER CELL LINES TO DOXORUBICIN

By

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Pharmacology
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ABSTRACT

Role of glutathione S-transferases in the resistance of human colon cancer cell lines to doxorubicin. Peter O. Beaumont, M.Sc., Department of Pharmacology, University of Toronto, 1997.

Doxorubicin (Dox) is effective in many types of cancer but has little activity against colorectal cancers. It is hypothesized that this is due to a multifactorial resistance mechanism in which the glutathione S-transferases (GST) may play a role. We have studied the relationship between GST isozyme expression, catalytic activity and Dox resistance in four human colon adenocarcinoma cell lines (HT-29, LoVo, SW620 & Caco-2) with the goal of modulating GST activity to overcome drug resistance. Levels of multidrug resistance associated protein (MRP) and P-glycoprotein (Pgp) were also determined. No relationship was observed between GST Pi expression and Dox resistance. A highly Dox-resistant cell line (Caco-2) showed a unique GST Alpha immunoreactivity not detectable in the other cell lines. GST class-selective inhibitors were not able to potentiate the cytotoxic effects of Dox in these colon carcinoma cell lines. The limited or absent expression of Pgp and MRP could not account for the 1,000-fold range of Dox sensitivities across the four cell lines.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to several individuals who have contributed to the research presented in this thesis.

For day-in day-out support, encouragement, insight and expertise I offer my most sincere thanks to my supervisor Dr. D.S. Riddick. Due thanks also go out to my co-supervisor Dr. M. J. Moore for bringing this research team together.

For their technical support and scientific contributions I would like to thank Peter McPherson, Chunja Lee, Anahita Bhathena, Kamran Ahmad, Michelle Payne, and John Giannone from the Department of Pharmacology, and also Jennifer, Pat and Trudy from the Princess Margaret Hospital / Ontario Cancer Institute.

Several individuals have provided equipment and materials which have made this research possible. I would like to thank Dr. K. D. Tew for providing a polyclonal GST Alpha antibody, Dr. A. J. Townsend for providing purified human GST A1 and A2 protein, Dr. D. Meyer for providing purified human GST A1, A2 and P1 protein and Dr. D.J. Waxman for providing several human GST oligonucleotide sequences. I would like to thank Dr. D.W. Hedley for allowing the quantitation of Pgp to be performed in his laboratory. For use of laboratory space and equipment I would like to thank Drs. B. Barber and G.J. Goldenberg.

Special thanks to Dr. A.S. Morgan of Terrapin Technologies for supplying us with samples of their GST inhibitor compounds.

I would like to acknowledge financial support for this research from the University of Toronto School of Graduate Studies and the Ontario Cancer Institute.

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LIST OF ABBREVIATIONS

α-MEM	alpha minimum essential medium
α-MEM-1X	α-MEM with antibiotic supplement
µg	microgram
µl	microlitre
µM	micromolar
ΔOD	change in optical density
5-FU	5-fluorouracil
ABC	ATP-binding cassette
ANOVA	analysis of variance
at-MDR	atypical MDR
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
BSO	buthionine sulfoximine
cDNA	complementary DNA
CDNB	1-chloro-2,4-dinitrobenzene
Chlor	chlorambucil
CHO	Chinese hamster ovary
CHP	cumene hydroperoxide
cm	centimetre
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNP-SG	dinitrophenyl-S-glutathione
DNR	daunorubicin
Dox	doxorubicin
DTT	dithiothreitol
EA	ethacrynic acid
EA-GSH	EA-glutathione conjugate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ETI	5,8,11-eicosatriynoic acid
ETYA	5,8,11,14-eicosatetraynoic acid
FBS	fetal bovine serum
G_0	resting cell phase
GPR	glycoprotein
GPX	glutathione peroxidase [selenium dependent]
GRAS	generally regarded as safe
GS-X	glutathione xenobiotic conjugate
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
HCl	hydrochloric acid
HEPES	hydroxyethane piperazine sulphate
HRP	horseradish peroxidase
$IC_{\#\#}$	concentration inhibiting ## % of growth or enzyme activity
kDa	kilodalton
Ki	inhibitory constant
mA	milliamperes
MDR	multidrug resistance
MDR1	gene for P-glycoprotein
mGST	mouse GST
ml	millilitre
mm	millimetre
mM	millimolar
MOPS	3-(N-Morpholino)-propanesulphonic acid
MRK-16	Pgp specific antibody
mRNA	messenger ribonucleic acid
MRP	multidrug resistance associated protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
N-SCLC	non-small cell lung cancer
NADP+	nicotinamide adenine dinucleotide phosphate (oxidized from)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide
O6AT	O^6 alkylguanine DNA transferase
OD	optical density
PBS	phosphate buffered saline
Pgp	P-glycoprotein
pKa	negative log of association constant
pmol	picomole
PNK	polynucleotide kinase
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SCLC	small cell lung cancer
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSBs	single strand breaks
SSC	150 mM NaCl/15 mM sodium citrate, pH 7.0
SSPE	150 mM NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.4
TE8	10 mM TRIS-HCl/1 mM EDTA, pH 8.0
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TER	Terrapin Technologies
TNT	20 mM TRIS/137 mM NaCl/0.1% (v/v) Tween-20 pH 7.6
Topo II	topoisomerase II
tPBO	trans-4-phenyl-3-buten-2-one
Tris-HCl	Tris (hydroxymethyl) aminoethane hydrochloride
V	volts
v/v	volume per volume
w/v	weight per volume
wt*p53*	wild type *p53* tumour suppressor gene

1. Statement Of The Research Problem

Drug-resistant colorectal tumours are a major health concern to many Canadians. Glutathione and the glutathione S-transferase (GST) enzyme family are implicated as factors in development of this resistance. This research study characterizes the presence and involvement of GSTs in colon cancer cell lines and investigates the feasibility of pharmacological manipulation of their activity, as a means to establish sensitivity to cancer cells resistant to the antineoplastic anthracycline doxorubicin.

2. Introduction

2.1 The Problem of Colorectal Cancer

Each year in North America, approximately 165,000 individuals are diagnosed with colorectal cancer. While many are successfully treated for their malignancy approximately 40% will succumb to the disease, making cancer of the colon and rectum the second leading cause of deaths related to cancer (Doyle, 1996). The primary treatment of this disease is surgical resection of the affected segment of the bowel which may prove curative. Most deaths, however, occur due to the metastases frequently associated with this disease. The risk of relapse and mortality due to metastases is related to size, differentiation, depth of invasion, the presence of vascular and neural invasion and, spread to the lymphatic system (Cunningham and Findlay, 1993). Adjuvant chemotherapy to treat these metastases and prevent relapse has proven mostly ineffective with nearly all agents studied (Moertel et al., 1990). The mainstay for chemotherapy for the last 30 years has been the antimetabolite 5-fluorouracil (5-FU) (Pratt et al., 1994).

2.2 Anthracyclines in Chemotherapy

The anthracyclines are a class of non-covalent DNA-binding drugs, of which the best known and most broadly used clinical agent is doxorubicin (Dox). This agent is used with significant efficacy either alone or in combination with other antineoplastics in the treatment of a variety of malignancies including acute leukemia, Hodgkin's disease, non-Hodgkin's lymphomas, breast cancer, sarcomas, ovarian cancer, small cell lung cancer, stomach cancer and bladder cancer. Despite this broad spectrum of anti-tumour activity, there is little efficacy of this agent toward colorectal cancers. The mechanisms behind the potent and relatively tumour-selective toxicity of the

anthracyclines have been studied extensively but are not, as yet, entirely clear. Several mechanisms have been implicated but none have proven to be ultimately responsible for cytotoxicity. There is evidence to support involvement of free radical toxicity due to redox cycling of the drug, intercalation of Dox to DNA, and disruption of membrane integrity due to Dox binding to membrane lipids (Doroshow et al., 1990; Awasthi et al., 1992; Pratt et al., 1994; Nielsen et al., 1996). Another mechanism which is strongly supported as a major contributor to Dox 's potent anti-tumour activity is the inhibition of the nuclear enzyme DNA topoisomerase II (Cummings et al., 1991). This enzyme, and the mechanism whereby Dox is believed to inflict its actions on it, are discussed under a separate heading below.

2.3 Multiple and Diverse Mechanisms of Drug Resistance

Resistance to anti-cancer drugs is a very frequent occurrence over the course of a clinical chemotherapeutic regimen. Many cancers display a pattern of an initial response to treatment but soon become refractory to the effects of both the agent(s) being employed and also to other structurally unrelated drugs. This ***acquired*** drug resistance occurs frequently and is the most common reason for failure of drug treatment (e.g. small cell lung cancer). Mechanisms of resistance have been characterized and attempts to modulate this resistance using chemosensitivity modulators are undergoing clinical trials. Cancers which display only a minimal response, or none at all from initiation of treatment, are referred to as having ***intrinsic*** drug resistance. Colorectal cancer is one of these intrinsically resistant malignancies.

There are several mechanisms postulated to be involved in both acquired and intrinsic drug resistance. Many of them are difficult to emulate in the laboratory as they

involve pharmacokinetic factors such as inability of the drug to achieve effective anti-tumour concentrations at the heart of a solid tumour. Poor vascularization of these solid tumours results in the inability of drugs to diffuse into the cancerous tissues, producing drug sanctuaries. In addition to poor diffusion of drug, the lack of effective angiogenesis to the core of a tumour also results in a body of cells which are poorly oxygenated and do not receive adequate nutrients. These cells are largely in the resting or G_0 phase and thus are not affected by a majority of drugs, or are unable to bioactivate certain agents. Other macroscopic factors include total tumour burden, tumour growth rate, patient immunocompetence, patient ability to tolerate drug side effects, drug scheduling and even circadian timing of chemotherapy.

The study of these clinical factors is clearly a vast field in its own right. It has been included in this discussion to provide a sense of the shear magnitude of the challenge faced by clinical oncologists, above and beyond the problems of cellular-based drug resistance mechanisms. The remainder of this discussion will not address these issues further and will focus solely on the involvement of the pharmacodynamic and subcellular means by which cancer cells develop drug resistance, and the steps being taken to overcome them. Much of this work will centre on the 'classic' multidrug resistance (MDR) phenotype. There are however, a number of novel mechanisms being evaluated for their potential involvement, either in acquired or intrinsic resistance, which deserve a brief overview.

One mechanism of drug resistance is an accelerated DNA repair. One study on sensitive and resistant P388 leukemia, measured single and double strand DNA breaks following exposure to Dox, and found respectively, 18- and 35- fold greater levels of DNA lesions in the sensitive line. While only a small amount of repair was

observed in sensitive lines 8 hours after exposure, 99% of the single strand breaks (SSBs) were repaired by this time point in the resistant line (Bankusli et al., 1989). A separate and more recent study comparing initially responsive small cell lung cancer cells (SCLC) to intrinsically resistant non-small cell lung cancer cells (N-SCLC) observed a significantly enhanced ability of N-SCLC cells to repair damage caused by cisplatin (Zeng-Rong et al., 1995). An explanation for these differences in DNA repair capacity has been offered as variation in expression of the ubiquitous DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase (O^6-AT). There is now independent evidence linking the expression of O^6-AT to drug resistance in other cancers including colon (Redmond et al., 1991; Mattern and Volm, 1995).

In addition to DNA repair enzymes, the tumour suppresser gene ***p53*** has been associated with drug resistance. The wild-type ***p53*** (wt ***p53***) nuclear phosphoprotein is known to bind to specific DNA sites and stimulates the downstream expression of genes that negatively control growth or invasion. In stressed cells, wt ***p53*** has been shown to induce apoptosis and/or cell-cycle arrest, which may facilitate DNA repair prior to replication. While the wt ***p53*** protein has been shown to repress the activity of the human ***MDR1*** gene in vitro (a major contributor to drug resistance discussed below), a mutant ***p53*** protein has been identified which exerts stimulatory effects on ***MDR1*** expression (Chin et al., 1992; El Rouby et al., 1993). The hypothesis that loss of normal ***p53*** function may confer drug resistance, has been tested and found to be correct in a number of cell lines. ***p53*** mutation has been associated with shorter overall survival and greater risk of death in B-cell chronic lymphocytic leukemia and ovarian carcinomas (van der Zee et al., 1995). Available studies in colon cancer indicate wt ***p53*** expression is relatively unchanged between sensitive and MDR

resistant sub-lines but do suggest mutation of the protein is responsible for the levels of ***MDR1*** observed (Mestdagh et al., 1995). A recent study on p53 indicates that inactivation of the wt protein in the absence of other genetic alterations leads to enhanced sensitivity to multiple chemotherapeutic agents rather than to increased resistance (Hawkins et al., 1996).

Another means by which tumours can display resistance to antineoplastics is through a loss of ability to undergo apoptosis at normally predetermined physiologic endpoints or following genotoxic insult. This inhibition of programmed cell death in malignant cells could result in both a growth advantage and a greater propensity to propagate DNA mutations. A study of colonic tissues has revealed that the transformation of normal epithelium to carcinoma may be accompanied by a progressive inhibition of apoptotic behaviour. In addition to altered ***p53*** expression in colorectal cancer the proto-oncogene ***bcl***-2 has also been investigated. This gene is known to prolong cell survival by inhibition of apoptosis. Normally, ***bcl-2*** is limited to the high growth crypt regions and expression is lost as cells differentiate and migrate to the luminal surface, where they will undergo programmed cell death as part of their expected turnover. In colon carcinomas, however, ***bcl-2*** has been found to be equally highly expressed in all regions of the malignant epithelium. This altered expression is speculated to be involved in the development of intrinsic drug resistance by decreasing the number of cells undergoing apoptosis in general, and also following genotoxic insult (Bedi et al., 1995).

The influence of tumour size on drug resistance through alteration in expression of drug-metabolizing enzymes has been investigated. For colonic tumour xenografts in mice, tumour size has been shown to impact significantly on the

expression of certain detoxification enzymes. Proteins from the GST family and the glucuronosyl transferases, which are involved in Dox metabolism are variably affected by tumour growth and both enzyme expression and catalytic activity may be related to overall tumour size (Massaad et al., 1994).

While glutathione (GSH), the major cellular thiol, is known to be involved in the metabolism of certain antineoplastics, other cellular thiols and detoxification mechanisms may also be important in drug resistance. Studies are being undertaken to assess the contributions of thioredoxin and metallothionine to drug resistance, and also the remarkable sensitivity displayed by certain tumours lacking these compounds (Yokomizo et al., 1995; Masters et al., 1996). Additionally, less mainstream drug metabolism enzymes such as transglutaminase have been observed to be upregulated in certain resistant tumours compared to sensitive parental lines (Mehta, 1994).

2.4 Primary Mediators of MDR

This overview of some of the less known potential drug resistance mechanisms sets the stage for our discussion of the classic group of detoxification enzymes and xenobiotic transporters. The following systems are thought to collectively contribute to drug resistance. Hopefully the reader can appreciate the vast array of subcellular responses that may be occurring both as a result of tumourigenesis and the pharmacologic tools applied to combat it. The concept of a single mediator of resistance is quite antiquated, with the multifactorial basis for this phenomenon now revealed. The key players in MDR appear to be: P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP), DNA topoisomerase II (Topo II) and GST. Each of these mechanisms will be outlined below, focusing on their relative

contributions to both intrinsic and acquired resistance in colorectal cancer. The involvement of glutathione and the glutathione S-transferase (GST) family of enzymes will be reserved for last, as the discussion will then shift from the 'basis of drug resistance' to a 'means of overcoming resistance', at least as far as GSTs are concerned.

2.4.1 Pgp

Over 20 years ago, it was discovered that certain cancers resistant to cytotoxic agents displayed collateral resistance often to a structurally diverse group of natural product antineoplastics (Ling and Thompson, 1973). Studies lead to the discovery of a 170 kDa surface glycoprotein (Pgp) whose gene when transfected into sensitive cells, was able to confer resistance to multiple drugs, including actinomycin D, etoposide, vincristine as well as, doxorubicin and other anthracyclines (Gros et al., 1986). Resistance to a broad spectrum of structurally dissimilar agents is termed 'multidrug resistance' (MDR), and the first gene thought to be responsible for it has been denoted *MDR1*. While humans have since been found to express a related *MDR3* gene, only the *MDR1* form appears to be active in drug resistance.

The product of the *MDR1* gene, Pgp, is a member of the evolutionarily conserved ABC (ATP-binding cassette) superfamily of membrane-bound transporters (Higgins, 1992). Current hypotheses refer to Pgp as a hydrophobic "vacuum cleaner", which removes potentially cytotoxic drugs from the plasma membrane before they reach the cytoplasm (Gottesman and Pastan, 1993). The basis for this ability to transport such a broad range of agents does not however lie in a common drug structure. The primary determinant of eligibility for transport is hydrophobicity and the substrate's ability to interact with the lipid bilayer (Zamora et al., 1988; Gottesman and Pastan, 1993). Transport is active, as efflux resulting in drug resistance is energy

dependent, and relies on an ATPase activity of the Pgp transporter (Endicott and Ling, 1989). The leap from a hydrophobic drug transporter to cellular resistance is made by comparing intracellular drug accumulation, Pgp expression, and extent of drug resistance. Using cell lines selected for Dox resistance, investigators have observed a relationship between drug accumulation, resistance, and Pgp. Although the extent of resistance or amount of altered accumulation is not always entirely accounted for, there is a trend toward elevation of Pgp levels leading to a decrease in drug accumulation and an increase in drug resistance (Dordal et al., 1994; Yang et al., 1995).

Pgp expression occurs to varying degrees in many normal tissues. Frequent expression in secretory and epithelial (barrier) tissues, that is typically localized to the apical surface of the cell membranes, suggests a physiological role for Pgp in cytoprotection from toxic xenobiotics and endobiotics (Bellamy, 1996). The human colon is a point of exposure to many such compounds. As a result, Pgp is expressed in up to 100% of tissue samples in studies on normal colorectal mucosa, polyps and pre-malignant dysplasias (De Angelis et al., 1995; Ikeguchi et al., 1995). The frequency of expression, however, decreases in malignant tissues and follows a continuing downward trend concurrent with the extent of de-differentiation taking place (Ikeguchi et al., 1995). Expression in cultured colon cell lines has proven to be quite variable (Satta et al., 1992). The extent of expression of Pgp in malignant tissues is often related to that in the corresponding normal mucosa; interestingly, the cancers originating from these tissues which normally express Pgp are often those which are most intrinsically resistant. Consistent with these findings, induction of differentiation with agents such as dimethylsulphoxide (DMSO) or sodium butyrate results in an

increased expression of Pgp and a subsequent increase in Dox resistance over parental lines (Mickley et al., 1989; Ho et al., 1994).

Pgp may have additional non-MDR related effects which are able to alter the intrinsic ability of cells expressing the gene to evade the effects of chemotherapy. One report provided evidence that colonic tumour cell populations expressing a higher Pgp level maintained a growth advantage over cells expressing a low Pgp level (Frommel, 1995). These data, along with the observation that cells in the leading edge of invading tumours and tumours with a high incidence of lymph node metastases express high levels of Pgp, support the hypothesis that Pgp contributes to a growth advantage and greater ability to repopulate following partially successful chemotherapy (Weinstein et al., 1991).

Evidence for Pgp involvement in the development of acquired resistance is quite strong in many tissue types. According to both ***in vitro*** and ***in vivo*** studies, Pgp expression may be elevated 35 to 70 % in tumours, following exposure to Dox (Sauerbrey et al., 1994; Volm et al., 1995b). While clinical results are not always in agreement, some studies have shown higher response rates in Pgp-negative tumours (breast/ovarian) and lower probability of remaining in complete clinical remission for tumours expressing Pgp (Sauerbrey et al., 1994; Veneroni et al., 1994; van der Zee et al., 1995).

2.4.2 MRP

A failure to detect Pgp in tumours and cell lines otherwise displaying the classic MDR resistance phenotype and decreased drug accumulation lead to the discovery of MRP. A differential hybridization approach identified an mRNA species, overexpressed in the Dox-resistant H69 human small cell lung cancer variant H69AR (Cole et al., 1992). The product of this gene is an ATP-binding integral membrane

glycophosphoprotein with an apparent molecular weight of 190 kDa (Almquist et al., 1995). The protein is primarily localized to the plasma membrane, although there is some evidence to indicate expression on nuclear membranes of drug-selected cells. This intracellular expression, however, is curiously absent in some transfectants (Abe et al., 1994; Zaman et al., 1994; Almquist et al., 1995; Breuninger et al., 1995). Transfection of the gene into MDR1 negative cells has been found to confer resistance to Dox (9.4-fold in NIH/3T3 cells), DNR, vincristine, etoposide, cholchicine and rhodamine, but not paclitaxel (Cole et al., 1994; Breuninger et al., 1995). The basis for this resistance appears to be a decreased intracellular accumulation of cytotoxic agents, as is found in Pgp-mediated MDR. Transport of these agents appears to be ATP-dependent and correlates directly with MRP expression (Jedlitschky et al., 1994; Leier et al., 1994; Zaman et al., 1994). While most studies have focused on active extrusion of compounds from the cell, others have speculated that translocation of the agents out of the nucleus into the cytoplasm or other internal compartments, may be equally important (Abe et al., 1994; Breuninger et al., 1995).

Western blots and immunohistochemistry have been used to determine the expression of MRP in normal and malignant tissues. MRP has been detected to varying degrees in many normal tissues and shows frequent expression in epithelia, muscle cells and macrophages, suggesting a similar excretory function to Pgp in protecting the organism against xenobiotics (Flens et al., 1996). The same study though, detected MRP in just 49 of 119 tumours, with 15 of 16 colon tumours being negative for the protein. The tumours which were found to express MRP were generally those from matched normal tissue which also expressed the protein. A separate, earlier study had detected MRP in 54 of 55 tumour cell lines with 7 of 7

colon tumours expressing the protein (Kruh et al., 1995). A third study detected MRP in 45% of gastric tumours (Endo et al., 1996). A fourth found the MRP gene expressed at levels considered to be significantly greater than control in 11 out of 26 (42%) colorectal carcinoma samples (Chuman et al., 1996). These conflicting data make it difficult to draw conclusions as to the relevance of MRP as a mediator of intrinsic drug resistance in the colon. It is, however, fairly clear, that when present, the protein does convey a resistance profile to tumours of many histologic origins, including that of the colon. Often the (over)expression of MRP correlates negatively with the tumour's sensitivity to Dox (Muller et al., 1994; Chuman et al., 1996; Endo et al., 1996; Kim et al., 1996). It is notable that the expression of MRP in a given tissue does not preclude expression of the other drug exporter, Pgp, and that the two may function in tandem to generate a greater degree of multidrug resistance (Brock et al., 1995).

Determination of the physiologic role for MRP and its endogenous substrates is not yet complete. Functional studies on the protein have revealed a number of relatively high affinity substrates and mediators which can modulate transport activity. The most readily transported substrates appear to be anionic conjugates of lipophilic compounds (Leir et al., 1996). The endogenous GSH-conjugate leukotriene C4 (LTC4) is, so far, the substrate of highest affinity. Other endogenous substrates include conjugated estrogens, the GSH conjugate of 4-hydroxynonenal and oxidized glutathione (GSSG) (Leier et al., 1994; Loe et al., 1996; Muller et al., 1996). Transport is not limited solely to GSH conjugates, as there is some evidence for transport of glucuronidated or sulfated compounds as well (Jedlitschky et al., 1996). Fascinating as it may be, the transport of these substances does not lead to any direct effects on drug resistance. The truly important findings are those surrounding enhanced efflux

of cytotoxic agents. While early studies in this area focused on the transport of GSH conjugated agents, recent studies have determined that MRP possesses the ability to actively transport, in an ATP-dependent manner, several unconjugated, lipophilic cytotoxic agents including DNR, etoposide and vincristine (Paul et al., 1996). This substrate specificity is overlapping with, but distinct from, that of Pgp, and includes both neutral or mildly cationic, natural product cytotoxic drugs and the anionic products of GSH conjugation (Paul et al., 1996). GSH depletion and repletion studies have proven the importance of GSH in drug transport by MRP through conjugation with this ubiquitous thiol, or at the very least, by maintenance of the cellular redox state, which may be required for effective MRP function (Versantvoort et al., 1995a; Paul et al., 1996). Whatever role it may perform, GSH is clearly involved in the generation of the MDR phenotype. Data implicating its involvement include: 1) GSH enhances transport of LTC4 and vincristine; 2) efflux of DNR requires GSH and; 3) cells expressing MRP extrude more GSH (likely as GSSG) than non-expressing cells (Zaman et al., 1995; Versantvoort et al., 1995a; Versantvoort et al., 1995b; Loe et al., 1996).

Initial studies which reported MRP as strictly a GSH-conjugate transporter, limited the hypothesis that decreased intracellular Dox accumulation could be conferred by MRP, as there is no evidence to suggest the existence of a Dox-GSH conjugate. Recent findings that MRP can extrude the anthracycline DNR, by a mechanism which requires GSH to be present but not necessarily conjugated to it, renew the theory that Dox is also transported by MRP but has not yet been studied completely (Paul et al., 1996).

2.4.3 Topoisomerase II

Alterations of DNA topological state, necessary for the consistent replication of eukaryotic cells, both normal or malignant, requires the involvement of a family of

enzymes known as DNA topoisomerases. These protein catalysts perform the task of resolving topological constraints in the processes of transcription, recombination, replication and chromosome partitioning during cell division [reviewed in (Hochauser and Harris, 1993; Watt and Hickson, 1994)]. The enzyme of interest, topoisomerase IIα (Topo IIα), has the primary task of introducing double strand breaks into DNA, creating a gap, through which an intact duplex is passed and then resealed. This enzyme is known to be the target of two types of antineoplastics: the epipodophyllotoxins (e.g. etoposide), and the anthracyclines (e.g. Dox and DNR). These agents have been reported to stabilize a reaction intermediate known as the "cleavable complex," which is composed of a drug-Topo IIα-DNA ternary complex. This non-functional unit disturbs both pre- and post-strand equilibria, causing in effect, increased DNA scission, detectable as DNA single or double strand breaks and DNA-protein cross-links (Fry et al., 1991). Interruption of Topo IIα activity results in the failure of re-ligation and subsequent fragmentation of DNA, which ultimately leads to cell death, the extent of which is proportional to the level of functional Topo II present. Topo IIα expression is also related to the rate of proliferation. Rapidly doubling normal cells, and tumours, have been found to express higher levels, while quiescent cells express lower amounts of the enzyme (Stammler et al., 1996). This heightened expression in tumours may be part of the basis for the selectivity generally seen for anthracyclines towards malignant tissues. It is not surprising then to find that drug-resistant cultured cell lines and tumour samples often have developed a means of resistance to the natural product cytotoxins. In addition to the typical MDR phenotype (decreased accumulation, increased efflux) observed following standard therapeutic regimens, cells treated specifically with intercalating agents may develop

an atypical MDR (at-MDR) specific to these drugs. Certain cancers have responded to chemotherapy by reducing their expression and activity of Topo IIα, reportedly by as much as 50% in colon carcinoma, as a means of resistance to these agents (Deffie et al., 1989; Rabier et al., 1991; Mestdagh et al., 1994; Yang et al., 1995). A second form of Topo II, Topo IIβ, is also being studied with respect to its role in the resistance of tumour cells to Dox and other antineoplastics (Brown et al., 1995). In addition to reductions in Topo II expression, many cancers have become resistant to anti-cancer drugs by altering the phosphorylation status (hyperphosphorylation) of the Topo II enzyme. This alteration renders this enzyme less susceptible to formation of the "cleavable complex" and hence reduces the number of lethal DNA lesions (Ackerman et al., 1985). Expression of at-MDR does not preclude the concurrent expression of typical MDR, which, as mentioned for MRP, can result in an even greater degree of drug resistance and a significantly greater challenge for clinicians.

2.5 Glutathione and the GST Enzyme System

2.5.1 Functions of GST in normal tissue

The primary resistance mechanism under evaluation in this study is the glutathione S-transferase enzyme superfamily. This group of enzymes catalyzes the conjugation of the cellular tripeptide γ-glutamylcysteinylglycine (glutathione, GSH) to a great number of electrophilic compounds. This ubiquitous low molecular weight thiol is present in cells at a concentration of 1 to 10 mM, and is capable of spontaneous nucleophilic attack on electrophilic centres. This conjugation reaction is generally considered to be detoxifying, and often renders compounds less reactive than their unconjugated parental form. This reaction can also serve as a marker for subsequent metabolism and excretion of the compound by other cellular pathways.

Although this reaction can occur non-enzymatically, the presence of GST allows it to proceed at a much accelerated pace (Gullick and Fahl, 1995). GST serves as a facilitator of the conjugation reaction by lowering the pKa of the thiol moiety of GSH. The effect of this is to stabilize the thiolate anion, the true nucleophile in the conjugation reaction (Graminski et al., 1989). The compounds that act as substrate for this reaction are structurally quite diverse, but share in common a degree of hydrophobicity. An example of such a compound (which is particularly relevant to this study), is the cytotoxic drug chlorambucil from the alkylating agent class of antineoplastics (Ciaccio et al., 1991). Additionally, free radicals generated by redox cycling following therapy with anthracyclines, such as doxorubicin, can be a significant source of endogenous toxicants which serve as substrates for GSH conjugation (Alin et al., 1985).

2.5.2 The Multiple Classes of GST

The GST enzyme system is believed to have been evolving since the appearance of aerobic organisms and has diverged considerably to combat the milieu of toxic endobiotics and xenobiotics encountered in the course of oxidative metabolism. There are at least four cytosolic and two membrane bound human GST classes (Mannervik et al., 1985; Mannervik et al., 1992). The main cytosolic forms Alpha, Mu and Pi will be the focus of this study. Little is known at this time about expression and activity of the more recently identified Theta class (Deakin et al., 1996), additionally little is known about the role of the microsomal class in drug resistance and hence these forms will not be appreciably addressed here. All cytosolic forms of vertebrate GSTs are comprised of two subunits and exist as either homo or heterodimers. The 24 to 29 kDa monomers each have an independently active glutathione binding G-site and hydrophobic substrate-binding H-site. These

monomers combine readily, however, only with members from within the same class, to form the fully functional enzyme (Tahir and Mannervik, 1986). This joining of monomers provides an added step of variability to this already diverse enzyme family. Within each GST class there can be multiple protein subunits, each encoded by a distinct gene. In humans there is only one Pi class subunit (GST P1); however there may be as many as five class Alpha genes encoding subunits (GST A1, A2, A3, A4 and skin GST 9.9) and 5 class Mu genes encoding subunits M1, M2, M3, M4 and M5 (Hayes and Pulford, 1995).

2.5.3 Expression Patterns of GST

These GST classes are expressed differentially throughout the body, and each class possesses a unique catalytic activity profile to cope with the range of toxicants it is likely to encounter. The Pi class has been detected in most tissues assayed thus far, and is clearly the most widely distributed. It is also frequently the most predominant GST expressed when more than one class is present, and typically the only GST expressed in the colon (Peters et al., 1989; Moorghen et al., 1991; Kelley et al., 1994). By comparison, GST Alpha isozymes show a more restricted distribution; most notably they are very highly expressed in liver. GST Mu forms are present mainly in muscles, testes, brain and lung. Interestingly, the *GSTM1* gene is deleted, resulting in a *GSTM1* null phenotype in up to 50% of the population which has been linked to a possible increased risk of lung cancer. To date, studies associating the *GSTM1* null phenotype with increased risk of colorectal cancer and/or patient outcome following chemotherapy are inconclusive (Zhong et al., 1993; Chenevix-Trench et al., 1995; Szarka et al., 1995). Recently, the GST expression patterns of a panel of 60 cell lines (of various human tissue origins), being maintained at the National Cancer Institute was published (Tew et al., 1996).

2.5.4 Class Selective GST Activity

Each of the GST classes possesses a unique catalytic activity profile. This activity signature can be exploited to both determine the contribution of individual classes to overall GST activity, and also possibly to provide enhanced drug targeting, as will be discussed below. GST Pi has traditionally, and in this study, been evaluated based on its activity towards the diuretic ethacrynic acid. Notable however, is its activity toward propenal and base propenals, which are compounds generated by oxidative processes and free radical reactions (Bernhane et al., 1994). GST Mu isozymes are particularly active with epoxides and *trans*-4-phenyl-3-buten-2-one (tPBO) (Comstock et al., 1994). In addition to its conjugative activity with powerful electrophiles such as alkylating agents (Ciaccio et al., 1991), the Alpha class of GSTs also possess a unique non-selenium-dependent glutathione peroxidase activity towards organic hydroperoxides, of which the typical agent of study is cumene hydroperoxide (Lawrence and Burk, 1976; Prochaska and Ganther, 1977). All human GSTs tested to date, except members of the Theta class (GST T1 and T2), have been found to have varying degrees of conjugative activity toward 1-chloro-2,4-dinitrobenzene (CDNB). As such, it is the substrate most commonly used for assessment of total GST activity (Habig et al., 1974).

2.5.5 Anthracycline Metabolism

The means by which the GSTs are able to influence the cytotoxicity of the anthracyclines and other free radical generating antineoplastics, are based on the proposed mechanisms of action of these drugs. In addition to the Topo II inhibiting activity of Dox, it is believed that redox cycling of the compound may generate sufficient free radicals to initiate an autocatalytic sequence of hydroxyl radical production and subsequent lipid peroxidation. There is currently evidence to both support and negate

this hypothesis. Studies in favour demonstrate that the production of hydroxyl radicals correlates with Dox sensitivity in some cell lines; that the less potent anthracycline 5-iminodaunorubicin does not generate free radicals and; that internalization of the drug is not explicitly required to produce antitumour activity (Tritton and Yee, 1982; Sinha et al., 1987; Doroshow et al., 1990). In addition, Dox is able to partition itself in the membrane by a factor of greater than 100-fold over the cytosol. The magnitude of this effect is 9-fold greater in sensitive than resistant cells (Awasthi et al., 1992). Studies against this hypothesis argue that insufficient free radical mediated DNA scission is occurring, at clinically achievable doses, to produce the lethality associated with this agent (Cummings et al., 1991).

Figure 1 illustrates the proposed pathways of Dox metabolism and free radical generation to produce toxic effects. The primary means of generation is enzymatic bioactivation of the quinone moiety of Dox, through an NADPH dependent one electron reduction to a semiquinone free radical. This compound is capable of transferring its free electron to molecular oxygen, returning it to the native Dox which can then initiate the oxidation-reduction cycle again. No particular enzyme has been identified as the specific Dox quinone reductase, although several are able to accept it as substrate, such as NADPH cytochrome P450 reductase in endoplasmic and sarcoplasmic reticulum; NADH dehydrogenase in mitochondria; xanthine oxidase in the cytoplasm and an as yet unidentified component in nucleii (Cummings et al., 1991). The superoxide anion produced as a result of redox cycling is not highly reactive in the cellular environment, but it is rapidly converted to hydrogen peroxide by superoxide dismutase. This peroxide is further reduced by an interaction with transition metals,

such as Fe^{2+}, to the hydroxyl free radical which is likely responsible for the lipid peroxidation observed (Doroshow et al., 1990).

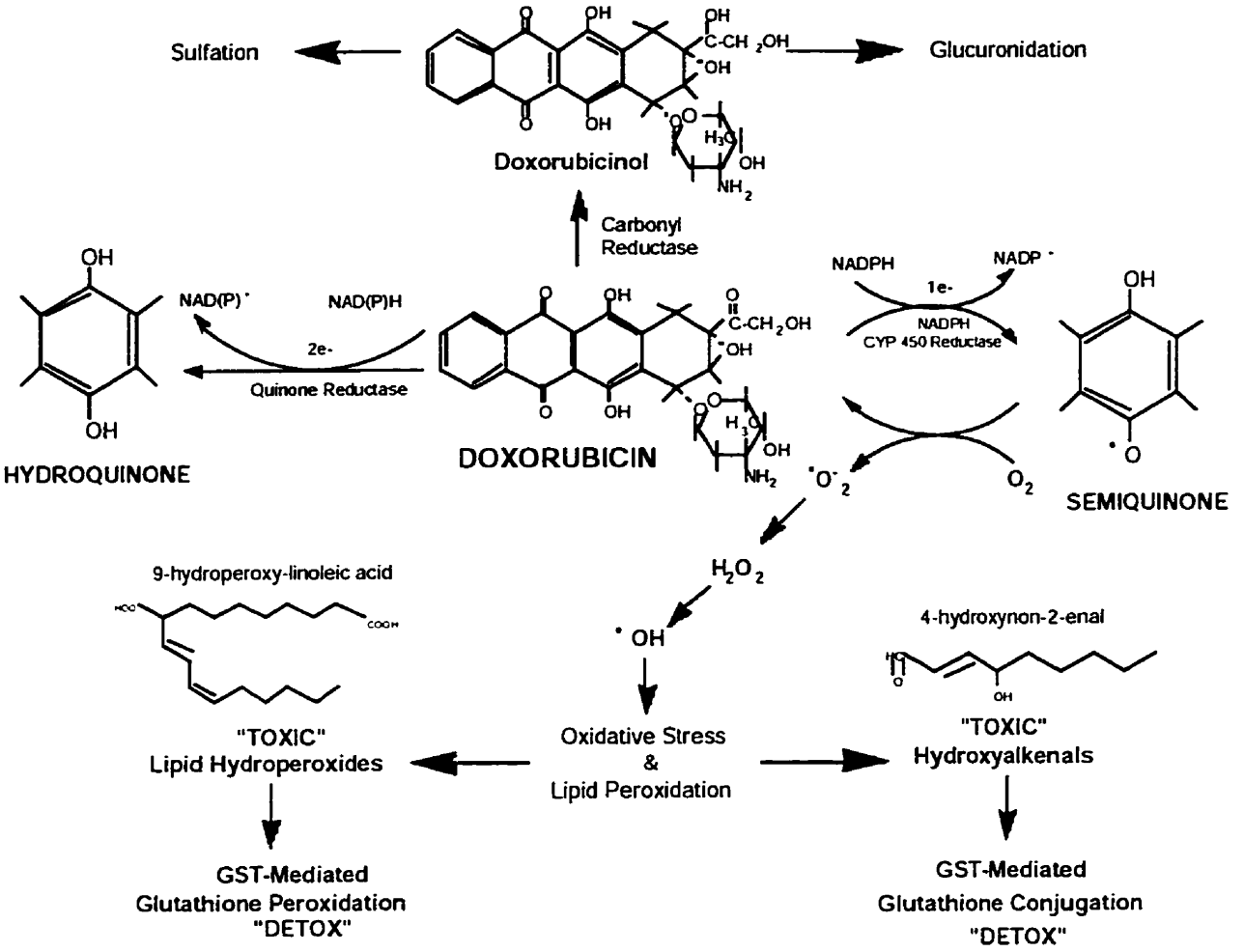

Figure 1: Major Pathways of Doxorubicin Metabolism

The mechanisms by which cells (tumour or normal) may respond to these radicals for self-preservation have been investigated. An increase in DT-diaphorase [a.k.a. NAD(P)H: quinone oxidoreductase] in response to quinone containing antineoplastic exposure has been observed in the HT-29 cell line (one of the cell lines used in this study). DT-diaphorase is a two electron bioreduction enzyme which produces a hydroquinone derivative of Dox and hence prevents the generation of toxic free radicals (Pan et al., 1995; Yao et al., 1996). Another contributing metabolic

mechanism to Dox resistance may be an increase in carbonyl reductase. This enzyme acts on a side chain of Dox to reduce the drug to its major metabolite doxorubicinol, which is ultimately glucuronidated or sulfated for excretion (Soldan et al., 1996).

Oxidative stress is an inherent part of normal aerobic metabolism in all eukaryotic cells. As such, a number of cellular defense mechanisms have evolved (in parallel with the GSH system) to cope with these stresses. Two well known enzymes active in this area are superoxide dismutase and catalase. In limited studies these enzymes have not been shown to be upregulated to deal with the increased stress load following Dox exposure (Sinha et al., 1987). The glutathione peroxidase (GPX) and GST system have however been found to be upregulated in response to Dox mediated oxidative stress in many cell types including colon (Mekhail-Ishak et al., 1989; Redmond et al., 1991). Transfection of GPX into MCF-7 breast cancer cells though, has failed to produce drug resistance despite 40-fold elevation of activity (Liebman et al., 1995).

Briefly, the mammalian GPXs are a family of selenium-dependent cytoprotective proteins with up to 5 members that are each highly homologous around the active site. The best studied member of the group is the 88 kDa cytosolic enzyme. The GPXs are capable of catalyzing the reduction of hydrogen peroxide or lipid hydroperoxides to water or lipid alcohols, respectively, using GSH as the reductant [reviewed in (Doroshow et al., 1990)]. There is some question though, as to whether these enzymes are effective in catalyzing the reduction of the phospholipid hydroperoxides which may be produced following free radical insult (Singhal et al., 1992). The peroxidase activity of the Alpha class of GSTs may however possess a

unique ability to detoxify such compounds in addition to its role in xenobiotic conjugation (Singhal et al., 1992). Evidence which further implicates GSTs in the development of drug resistance will be discussed.

2.6 Evidence for Involvement of GST in Drug Resistance

Whether it functions alone or in concert with other putative mechanisms, several lines of experimental evidence support the involvement of GSTs as mediators of drug resistance, including: 1) GSTs catalyze the GSH conjugation of specific anti-cancer drugs; 2) patterns of GST overexpression in colorectal cancer; 3) effects of GSH depletion on drug resistance; 4) effects of GST transfection into normally non-expressing cell lines; 5) results of anti-sense GST DNA studies; and 6) results of studies on tumour sensitization following pharmacologic inhibition of GST. The manner in which these studies support or negate the involvement of GSTs in drug resistance is outlined below.

2.6.1 Conjugation of antineoplastics with GSH catalyzed by GST

Evidence for involvement of the GSTs in drug resistance for certain antineoplastics is quite strong. Chlorambucil, melphalan, cyclophosphamide, thiotepa and busulfan are all conjugated directly to GSH by GST as part of cellular metabolism (Dirven et al., 1994; Hall et al., 1994; Dirven et al., 1995; Gibbs et al., 1996). Dox, however, is not reported to undergo such conjugation. Instead the toxic products of lipid peroxidation, such as the hydroxyalkenals, are reported to be detoxified by a GST Pi mediated process involving GSH (Alin et al., 1985).

2.6.2 GST Overexpression Patterns

Numerous studies comparing untreated tumours in the colon with matched normal mucosa have shown an elevation in tumour GST Pi expression, and a concurrent increase in catalytic activity (Mekhail-Ishak et al., 1989; Clapper et al., 1991;

Moorghen et al., 1991; Ranganathan and Tew, 1991; Redmond et al., 1991; Peters et al., 1992). The amount of increase in protein expression is typically small (1.5 to 2.3 fold) but significant, as is the change in catalytic activity. The contribution of GSTs Alpha and Mu to drug resistance is not clear, as their expression is often either decreased or not correlated with development of colon cancer (Ranganathan and Tew, 1991; Peters and Roelofs, 1992). Some studies have reported variable expression and even downregulation of GST Pi in non-colonic tissues (Klone et al., 1990; Sasano et al., 1993). No tumour examined thus far has expressed GST enzymes that were not already present in matched normal tissue (Campbell et al., 1991; Kelley et al., 1994). Studies in a variety of cancers (breast, ovarian, lymphoma, lung), attempting to correlate tumour characteristics such as Duke's stage of differentiation, histologic type, size, patient age and patient gender with resistance mechanisms and response to therapy have generally proven futile (Berhane et al., 1995; Frierson et al., 1995; Inoue et al., 1995; Wrigley et al., 1996). Limited studies focusing on the colon have, however, been able to show a significant correlation between response to chemotherapy or patient outcome and GST expression or activity, with high GST levels being associated with poorer prognosis (Okuyama et al., 1994; Mulder et al., 1995).

Studies on normal and malignant tissues provide evidence for GST involvement in the intrinsic drug resistance seen in the colon. Exposure of immortal cell lines or tumours to sub-lethal doses of Dox produces fairly consistent elevations in GST expression, as such a number of GST overexpressing Dox-selected cell lines have been generated for use in studying drug resistance (Rabier et al., 1991; Stelmack and Goldenberg, 1993). Cell lines originating from the colon and other

tissues routinely display an approximately 2-fold elevation in GST protein expression, and a corresponding increase in the dose of Dox required to reach end-points of cytotoxicity such as IC_{50} (Chao et al., 1992; Perry et al., 1993; Volm et al., 1995b). In a patient with SCLC receiving a Dox-containing regimen, a biopsy was used to establish a primary cell culture at diagnosis and at subsequent relapse. This culture was subsequently assessed for GST catalytic activity. It was observed that as the tumour progressed from sensitive to resistant, there was a distinct (2-fold) increase in GST activity, accompanied by a 1.5 fold increase in resistance (de Vries et al., 1989). Studies such as this provide a crucial link between the effects observed on immortal cell lines selected for resistance, and the types of changes occurring in the clinical setting.

2.6.3 GST Transfection Studies

Additional evidence for involvement of GSTs in drug resistance is obtained from studies where GST P1-1 cDNA has been transfected into drug sensitive cell lines, which generally do not express the enzyme or only express it to minimal levels. These studies, while not showing entirely positive results, do provide insight into the complexities of the development of drug resistance in tumour cells. One study, which transfected a GST P1 expression vector into MCF-7 wild type cells, was able to confer GST Pi expression at a level similar to Dox-selected MCF-7/ADR cells, but was unable to alter drug sensitivity (Moscow et al., 1989). A separate study, however, successfully generated two NIH 3T3 drug resistant sub-lines following transfection with the GST Pi vector. Unlike MCF-7 these cells displayed both an elevated GST Pi expression, and a degree of drug resistance (1.8 and 3.0-fold) which was roughly associated with it (Nakagawa et al., 1990). In addition to GST Pi, another acid transferase, hGST 5.8, which is the human orthologue of mouse mGSTA4-4, has been identified and

reported to posses strong activity toward 4-hydroxyalkenals and lipid hydroperoxides (Singhal et al., 1994). This enzyme has been transfected into Chinese hamster ovary (CHO) cells, resulting in a 40 % reduction in membrane peroxidation and a 2-fold increase in Dox resistance (He et al., 1996). These modest and inconsistent responses to Dox conferred by GST transfection indicate that while GSTs are likely *involved* in the development drug resistance, expression of the enzyme alone is not always sufficient to produce it. Hence, involvement of other cellular protection systems is a likely necessity. In some cases, as is observed with the NIH 3T3 and CHO cells, the necessary systems may be constitutively expressed, while in others, the stresses of drug exposure are required to induce their expression.

2.6.4 GSH Depletion studies

An area which has received much attention in the quest to elucidate the mechanisms behind drug resistance is the effect of GSH depletion on response to chemotherapeutics. To date, most work has focused on the pharmacologic depletion of GSH by preventing its synthesis, rather than accelerating its utilization. This effect is achieved by inhibiting the first enzyme of GSH synthesis, γ-glutamylcysteine-sythetase, using the selective inhibitor L-buthionine (S,R)-sulfoximine (BSO) (Griffith and Meister, 1979). Numerous studies have provided evidence that depletion of cellular GSH can sensitize drug-resistant cells (up to 12.9-fold) and, to a lesser extent, parental cells (approx. 1.5-fold) to a variety of antineoplastics, including Dox (de Vries et al., 1989; Lai et al., 1991; Meijer et al., 1991; Chao et al., 1992; Xu and Singh, 1992; Kisara et al., 1995a; Kisara et al., 1995b; Schneider et al., 1995; Ali-Osman et al., 1996). As this agent inhibits the enzyme necessary for synthesis and does not deplete GSH directly, it generally must be administered 24 hours prior to drug

exposure, and is most effective if present throughout the culture period for the cytotoxicity assay. Concentrations of 25 to 100 μM administered in this fashion have been shown to cause up to 95% depletion of free GSH in colon and other cell lines (Lai et al., 1991; Ali-Osman et al., 1996). Some studies have found BSO administration alone is sufficient to return sensitivity to resistant cell lines, while others have found additional drug-resistance mediators are often necessary to achieve parental cytotoxicity profiles, indicating the involvement of multiple resistance mechanisms in these cell lines (Lai et al., 1991; Kisara et al., 1995b).

2.6.5 Anti-sense GST cDNA Studies

Evidence for the involvement of GSTs (specifically the Pi class) in drug resistance also stems from recent studies employing anti-sense cDNA technology, in both parental and drug resistant cell lines. Using M7069 colon cancer cells which possess intrinsically high GST Pi expression (but no Pgp or MRP), along with the resistance-selected sub-line M7069/ADR, Ban et. al. demonstrated that decreasing GST Pi expression through the use of antisense technology, was an effective means to confer sensitivity to these cells (Ban et al., 1996). A 50% reduction in GST Pi expression was associated with a 3.3 fold increase in sensitivity in the parental line and 4.4-fold increase in the drug-resistant line. The authors suggest that these data provide, for the first time, evidence that GST is a ***cause*** of intrinsic resistance, and that it may be more important than Pgp in the early stages of chemotherapy.

The last form of experimental evidence which supports the involvement of GSTs in antineoplastic resistance, comes from studies in which there is an attempt to inhibit the functions of GSTs in resistant cells using pharmacologic agents. The idea being, that if an agent which is known to inhibit GST function, returns drug sensitivity to

a resistant cell line, then GSTs must be part of the basis for drug resistance. The corollary from this, which will be discussed in depth in this paper, is that, class-selective inhibition of overexpressed GSTs in clinical specimens may be a feasible means to overcome MDR.

2.7 The GST Inhibitor Hypothesis

2.7.1 Ethacrynic Acid

2.7.1.1 In vitro studies

The GST inhibitor ethacrynic acid (EA), a phenoxyacetic diuretic, (Ahokas et al., 1985) has been investigated as a possible modulator of drug resistance towards a variety of antineoplastics, including: thiotepa, cyclophosphamide, chlorambucil, melphalan, mitomycin C and doxorubicin (Tew et al., 1988; Nagourney et al., 1990; O'Dwyer et al., 1991; Xu and Singh, 1992; Chen and Waxman, 1995) . It is believed that the ability of EA to reverse drug resistance is due to its inhibitory effect on GST, however, the mechanism by which it performs this function is complex and not fully understood. EA interacts with GST as a substrate, as a non-substrate ligand and the GSH conjugate of EA (which can be formed spontaneously or via a GST mediated reaction), can also inhibit the enzyme. All forms of this conjugation reaction are reversible, though the EA-GSH conjugate is a competitive inhibitor of GST, while EA alone is non-competitive with respect to CDNB. EA-GSH has been shown to be an order of magnitude more potent than EA at inhibiting GST conjugation activity with reported K_is of 1.5 and 11.5 μM respectively (Awasthi et al., 1993; Shen et al., 1995). EA has been shown to bind irreversibly to GST, but there is no data to indicate that such binding has a significant effect on GST activity (Ploemen et al., 1990). The most convincing data for return of sensitivity due to GST inhibition, comes from ***in vitro*** cell survival studies on cell lines or tumours where various forms of GST are

overexpressed. Combining the alkylating agent chlorambucil (which is known to be conjugated to GSH primarily by GST Alpha and to some extent by GST Pi), with EA has been shown to confer a clear enhancement of toxicity compared with the use of the antineoplastic alone (Tew et al., 1988; Xu and Singh, 1992; Chen and Waxman, 1995). At this time there is, however, only limited data for potentiation of toxicity involving Dox-EA combinations (Nagourney et al., 1990).

2.7.1.2 Clinical Studies

Based on the ***in vitro*** successes of EA, ***in vivo*** and phase I clinical trials are underway to assess if similar potentiation effects can be seen in animals and human subjects. HT-29 xenografts in scid mice have been shown to be sensitized by EA to melphalan. Additionally, human pharmacokinetic studies indicate that GSTs are inhibited ***in vivo*** at reasonably attainable doses (Clapper et al., 1990; O'Dwyer et al., 1991; Lacreta et al., 1994). Results of trials assessing for increases in progression free or overall survival subsequent to GST modulation therapy are not yet available. Despite these reported ***in vitro*** successes, EA has some finite limitations including: a) EA both induces and also inhibits GST Pi, inhibits NAD(P)H oxidoreductases, and induces dihydrodiol dehydrogenase; b) neither EA nor EA-GSH are selective for any particular GST class; c) EA depletes GSH and reacts with other cellular sulfhydryls in both tumour and normal tissues; and d) clinically, EA causes marked diuresis and concomitant electrolyte imbalances in humans [reviewed in (Ciaccio et al., 1995)].

2.7.2 Alternatives to EA

Because of the shortcomings of EA, many investigators are searching for other pharmacologic agents which may be more selective for the particular GST classes overexpressed in a given tumour (GST Pi in the colon), and which do not possess such broad, non-selective enzyme induction and inhibition properties. One of the

agents under study as a potentially, clinically useful GST inhibitor is sulfasalazine. This agent, a drug commonly used in the treatment of inflammatory bowel disorders, is an effective inhibitor of GSTs, boasting IC_{50}s of 20, 0.2 and 13 μM for GSTs Alpha, Mu and Pi respectively. Like EA, it is an approved therapeutic agent in humans, which is appealing from a regulatory standpoint, if and when clinical trials are warranted (Awasthi et al., 1994a; Gupta et al., 1995). Also under investigation, are the acetylenic fatty acids. Two in particular, 5,8,11-eicosatriynoic acid (ETI) and 5,8,11,14-eicosatetraynoic acid (ETYA) have been studied in human liver and display IC_{50}s of 18 and 108 μM toward CDNB (class-selective studies have not been carried out thus far). Unlike EA, these latter two agents are not approved for human therapeutic use (Datta and Kulkarni, 1994). A third agent recently reported to have inhibitory capabilities on GSTs is eugenol. This naturally occurring phenolic compound is a major component of clove oil and is also present in oils of cinnamon, basil and nutmeg. It has been granted GRAS-status (Generally Recognized As Safe) and is hence, acceptable for human consumption. Few data are available thus far other than IC_{25} values of 0.8 mM eugenol for GST Pi, 0.4 mM isoeugenol methyl ether for GST Mu and 0.6 mM eugenol methyl ether for GST Alpha against CDNB. Unique among the agents studied thus far, eugenol (100 μM), when combined with tyrosinase and GSH, is able to produce irreversible GST inhibition that nearly completely halts GST P1-1 activity and possesses significant inhibitory effects against GST A1-1 and GST M1a-1a. It is believed that a covalent bond may be formed between the two subunits of GST P1-1, as a basis for the irreversible effect (Rompelberg et al., 1996).

Lastly, a bifunctional inhibitor has been synthesized by Hidaka and coworkers which inhibits both GST and Pgp activity (Terasawa et al., 1992). This

isoquinolinesulfonamide derivative is a calmodulin antagonist and its double action is able to reverse 70-fold resistance in Dox-selected ovarian carcinoma cells. The agent is however highly toxic to Dox-sensitive cells and hence is not suitable for clinical use (Maeda et al., 1993). We await further findings in these areas to help overcome resistance due to GST overexpression.

Another avenue of GST inhibition, which will be the focus of much of this work, is that of GST inhibition through the use of GSH analogues.

2.8 The Glutathione Analogue Hypothesis

The quest to develop a more potent and class-selective GST inhibitor has recently focused on the development of several GSH analogues. Modifications of the basic GSH structure has been used to achieve greater affinity of the analogue to the GSH-binding G-site on the GST enzyme. Building on the independent findings of the research teams of Adang and Askelof, a number of novel inhibitors have been developed (Askelof et al., 1975; Adang et al., 1990). Askelof had originally shown that the potency and selectivity of S-functionalized GSH analogues as inhibitors of GST activity varied with the length of n-alkyl groups. Adang more recently produced a series of analogues in which the C-terminal glycine of GSH had been replaced by different amino acids. Presently, at least two investigative teams are using these data to generate novel analogues, which may be used to inhibit the GSTs in drug resistant cells overexpressing them.

2.8.1 R-Hex based Analogues

One group has used the R-HEX {(R)-5-carboxy-2-γ-(S)-glutamylamino-N-hexylpentamide} compound synthesized by Adang et al (1990) as their lead compound for further design of ***in vivo*** active inhibitors. To obtain a more potent

inhibitor the N-hexyl moiety was replaced by N-2-heptyl and additionally, to increase uptake of the compounds *in vivo,* the 5-carboxy group was esterified with ethyl (Et-R-Hep) and dodecyl (Do-R-Hep) groups (Ouwerkerk-Mahadevan et al., 1995). Using bromosulphothalein (BSP) as substrate, the compounds were found to posses inhibitory abilities toward purified human GST Alpha and Mu isozymes, in the low micromolar range and, were observed to be competitive with respect to GSH. The compounds were ineffective however at up to 500 μM against GST P1-1. In these *in vitro* studies the unesterified versions were more potent than their respective esterified compounds. This situation though, was reversed *in vivo.* The most potent *in vivo* inhibitor of these compounds, Do-R-Hep unfortunately proved to be too toxic for further study. The Et-R-Hep compound, however, was well tolerated when administered to rats, with no obvious toxicity and displayed significant decreases in BSP conjugation to GSH.

2.8.2 Combined S-functionalization and C-terminal modifications

The second research group, based at Terrapin Technologies (South San Francisco, CA) combined both S-functionalization and C-terminal modification to produce their own series of GST inhibiting GSH analogues. Initial studies in this area employed an analogue strategy for screening, in order to target a manageable number of compounds which would represent a broad sampling of the potential diversity of GST inhibitors (Kauvar, 1992). Parameters such as hydrophobicity, size, and electronegativity, which are known to be relevant to GST binding, were applied when selecting functional groups to substitute in the GSH moiety. Of the numerous compounds generated, several were found to have K_is below 1.0 μM, exhibit greater than 5-fold selectivity between isozymes, inhibit greater than 90% of GST activity, and

were readily obtainable at over 90% purity. Additionally the compounds were found to specifically interact with the GST active site in a competitive manner (with respect to GSH) to produce their effects (Flatgaard et al., 1993). A later study further characterized these and other GSH analogues and also generated diethyl ester versions of them. This modification, as observed by Ouwerkerk-Mahadevan et al (1995), is necessary to allow rapid penetration of the cell membrane by the compound, as the free acid versions of the GSH analogues are unable to do so (Lyttle et al., 1994). Data indicate that the diethyl ester moiety is readily cleaved in the cytosol of colon cells (Morgan et al., 1996).

Diethyl Ester Form	Free Acid Form	Chemical Name	K_is (uM)			
			P1	A1	M1a	M2
TER 183	TER 143	Glu-S-**Octyl**-Cys-Gly	1.9	0.27	1.2	ND
TER 199	TER 117	Glu-S-**benzyl**-Cys-**phenyl**-Gly	0.4	20	25	31
TER 206	TER 211	Glu-S-**βnaphthyl**-Cys-Gly	1.2	4.2	0.01	1.5

Table 1: List of GST Inhibitors

Compounds listed above were provided for study by Terrapin Technologies (South San Francisco, USA).K_is obtained from (Morgan et al., 1996). All compounds are based on the reduced GSH structure, with substitution of various functional groups. These particular agents were selected based on their specificity for the three GST classes (Flatgaard et al., 1993; Lyttle et al., 1994).

2.8.3 Chemosensitization

To date, only the Terrapin compounds have been assessed for their ability to potentiate the effects of antineoplastics. Three of these new GSH analogues (TER 117, 143 & 211) and their diethyl esters (TER 199, 183 & 206), which are selective for GSTs Pi, Alpha and Mu respectively, have recently been assessed for their ability to modulte drug sensitivities in cell lines (Morgan et al., 1996). The chemical modifications and K_is for inhibition of recombinant purifed enzyme are listed in Table 1. Using the HT-29 human colon cancer cell line and an ethacrynic acid-sensitive sub-clone (HT4-1), it was observed that TER 199 (25 μM) could enhance toxicity of the alkylating agent chlorambucil by greater than 2-fold. The extent of potentiation seen by this and other analogues correlated positively with the potency of parent compounds as inhibitors of GST P1-1, the dominant isozyme in this cell type. Application of the free acid GSH analogues displayed neither toxicity when used alone (up to 200 μM) nor potentiation when used in combination with alkylating agents. A combination

therapy of TER 199 and melphalan was found to retard tumour growth in HT4-1 xenografts implanted in scid mice, more than melphalan alone. Results of Dox used in combination with TER 199 and other analogues became available only after commencement of our study. Using the HT4-1 colon cancer cell line and a standard clonogenic assay, it has been observed that TER 199 and TER 183 can provide a modest (1.1 to 1.2-fold) enhancement of toxicity to Dox (Morgan et al., 1996).

2.8.4 Further Evaluation of GSH Analogues

While these studies provide encouraging evidence that GST-inhibiting compounds may be able to potentiate the effects of certain antineoplastics, there remain many basic questions about GST involvement in drug resistance and how to overcome it. Some of these questions will be addressed here. Most importantly, while these compounds may be active ***in vitro***, and are believed to be returned to their active state following de-esterification in the cytosol, there is as yet, no evidence to indicate that GST inhibition is actually occurring ***in vivo***. Additionally, the published data reflect the effects of these agents on a limited number of cell lines, (only one from colon) all of which express GST Pi as the predominant enzyme. Study of a greater number of lines may provide more insight into the type and extent of effects which may be occurring, especially those related to variable GST enzyme expression. A search to elucidate the answers to these questions on GST involvement in colorectal cancer, may also provide clues to how the various resistance mechanisms interact to produce clinical MDR.

Compounds selective for GST P1-1, A1-1 and M1a-1a have been made available for our study to evaluate the involvement of GSTs in the drug resistance of human colon cancer cell lines.

2.9 Specific Research Goals

The extent of the problem of colorectal cancer has been demonstrated, as well as the virtual futility of using chemotherapy as a primary, treatment for it. The rather extensive list of potential sub-cellular mechanisms of drug resistance has been discussed, at the very least to the extent of stressing how extremely naive it would be to deal with drug resistance as a one-dimensional problem. There is substantial evidence for the involvement of GSTs in the resistance displayed by many cancers, and also evidence against it in others. This study will focus almost exclusively on cancer of the colon, exploring the question of whether GST enzymes play a role in the intrinsic resistance expressed by these cells towards doxorubicin. We will do this by determining the level of GST Alpha, Mu and Pi mRNA and protein in four independent colon cancer cell lines, that represent a broad spectrum of drug sensitivities. Additionally, the catalytic activity of these GSTs will be determined in order to relate expression to function. The study will digress from GST only to address the question of the extent to which other classic MDR mediators (Pgp and MRP) contribute to intrinsic resistance of colon cancer cells to Dox. The GST inhibitors discussed above (EA, TER 183, TER 199 & TER 206) will be assessed for their ability to inhibit the total and/or isozyme-selective GST activity present in these cell lines. This assessment will be conducted under in vitro assay (free acid forms) and also in intact cells (diethyl ester forms). These findings will ultimately be compared against the ability of the GST inhibitors to potentiate the cytotoxic effects of doxorubicin as assessed by cell survival assays.

3. Hypotheses

1. Adenocarcinomas of the colon express varying levels of GST isozymes.

2. Overexpression of GST enzymes and elevated activity are related to doxorubicin resistance in colon cancers.

3. Inhibition of GST with class-selective GST inhibitors sensitizes resistant cells to the cytotoxic effects of doxorubicin.

4. GST works in conjunction with MRP and/or Pgp in producing drug resistance in colon cancer.

4. Objectives

1. Assay levels of GST Alpha, Mu and Pi protein and mRNA in four colon carcinoma cell lines (Caco-2, HT-29, LoVo and SW620).

2. Determine total and class-selective catalytic activity of GSTs in these cell lines.

3. Determine the level of other resistance mechanisms (MRP and Pgp) in these cell lines.

4. Determine the relative resistance of the four cell lines to doxorubicin.

5. Attempt sensitization of cell lines to doxorubicin with class- selective GST inhibitors.

5. Methods

5.1 ***Drugs & Reagents***

Doxorubicin HCl was obtained from Pharmacia / farmitalia Carlo Erba (Italy). Three glutathione analogues, Terrapin 183 [γ-glutamyl S-(octyl)cysteinyl glycine diethyl ester], Terrapin 199 [γ-glutamyl S-(benzyl)cysteinyl phenylglycine diethyl ester] and Terrapin 206 [γ-glutamyl S-(β-naphthyl)cysteinyl glycine diethyl ester] (TER183, TER199 and TER206) and their respective free acids TER143, TER117 and TER211, used as GST isozyme-selective inhibitors, were provided for this study by Dr. Amy S. Morgan, Terrapin Technologies Inc. (South San Francisco, CA). ***Trans***-4-phenyl-3-buten-2-one (tPBO) was purchased from the Aldrich Chemical Company (Milwaukee, WI). Enhanced Chemiluminescence reagents and horseradish peroxidase antibody conjugates for western blotting and [γ-^{32}P] ATP (specific activity > 5,000 Ci/mmol; radioactive purity > 95%) were purchased from Amersham Life Sciences (Arlington Heights, IL.). Electrophoresis and blotting equipment and reagents were purchased from Bio-Rad Laboratories (Hercules, CA), Mallinckrodt Specialty Chemicals Co. (Chesterfield, MO) and Sangon Ltd. (Scarborough, ON). Dimethylsulfoxide (DMSO), sodium dodecylsulfate (SDS), glycine, Tris (hydroxymethyl) aminomethane (Tris), and dithiothreitol(DTT) were purchased from Caledon Laboratories Ltd. (Georgetown, ON). Lyophilized sterile trypsin was purchased from Difco Laboratories (Detroit, MI). Cell culture antibiotics and fetal bovine serum were purchased from Life Technologies (Gaithersburg, MD). Tri-Reagent® for RNA isolation was purchased from Molecular Research Center Inc. (Cincinnati, OH). T4-polynucleotide kinase was purchased from Pharmacia Biotech (Uppsala, Sweden). Ethacrynic acid, Tween-20,

thimerosal, chlorambucil, Dulbecco's PBS, N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulfonic acid] (Hepes), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), Ponceau S-concentrate, cumene hydroperoxide, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione, GSSG reductase and NADPH were purchased from the Sigma Chemical Company (St. Louis, MO). Alpha minimum essential medium (α-MEM) and sterile phosphate-buffered saline (PBS) were purchased from the University of Toronto Media Preparation Service.

5.2 Antibodies and Purified Proteins

Rabbit polyclonal antibodies raised against human GST Alpha, Mu and Pi were purchased from Biotrin International (Dublin, Ireland). An additional rabbit polyclonal antibody against human GST Alpha was obtained as a gift from Dr. Ken Tew, Fox Chase Cancer Center (Philadelphia, PA). A mouse monoclonal anti-human MRPm6 antibody was purchased from Kamiya Biomedical Company (Tukwila, WA). Purified human GST A1-1 and A2-2 were a gift from Dr. Alan J. Townsend, Bowman Gray School of Medicine, Wake Forest University (Winston-Salem, NC). Purified human GST A1, A2, and P1 were a gift from Dr. David Meyer, University College London (London, UK).

5.3 Cell Culture

5.3.1 Cell lines and culture techniques

Four human colon cancer cell lines which represented both a broad range of sensitivities to Dox (Lai et al., 1991; Chao et al., 1992; Peters and Roelofs, 1992; Veneroni et al., 1994) and which were also known to perform the necessary mitochondrial metabolism of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] required for the colourimetric cytotoxicity assay (Alley et al., 1988; Peters and

Roelofs, 1992) were chosen for this study. All cell lines were purchased from the American Type Culture Collection (ATCC) (Rockville, MD), and shipped by air express on dry ice. On receipt, cells were thawed by rapid agitation in 37 °C water bath and diluted according to product sheet specifications, into gamma irradiated 80 cm^2 tissue culture flasks (Nunclon, T80). All cell culture work was carried out under sterile conditions in a SterilGARD (Baker Co., Sanford, MA) laminar flow tissue culture hood. Three lines (Caco-2, HT-29 and LoVo) were derived from culture of human colon adenocarcinomas while the fourth (SW620) was obtained from a lymph node metatasis of human colon adenocarcinoma. A fifth line, LS180, also of colonic adenocarcinoma origin, was cultured briefly for generation of cytosolic fraction samples, but was not used for studies on cell growth or drug resistance.

All cell lines were maintained in alpha-minimum essential medium (α-MEM) supplemented with 10% FBS in either T25 or T80 flasks. Cells were grown in an incubator at a constant 37°C in a humidified atmosphere containing 5% CO_2. Subculture was performed approximately weekly, at dilutions ranging from 1:3 to 1:30. Variation in subculture was based on individual cell line growth properties and the schedule of pending cell culture based experiments. To perform subculture, near confluent flasks were retrieved from the incubator and all growth medium was aspirated with an autoclaved glass pasteur pipette. Residual medium was removed by washing with 13 ml sterile PBS. PBS was then aspirated and approximately 2 ml of sterile trypsin was added. Cells were then placed back in the incubator for 10 min to allow for trypsinization. At the end of this brief incubation, cells were returned to the laminar flow hood and 7 to 13 mls (depending on desired subculture density) of medium was added to halt the action of trypsin. The culture medium was then mixed

by repeated up and down pipetting to obtain a near single cell suspension. Aliquots of 0.5 to 4 mls were dispensed into T80 flasks already containing an appropriate amount of α-MEM to produce a final flask volume of 12 ml.

To control against bacterial and fungal infections, one flask per cell line was grown in α-MEM containing single strength antibiotic/antimycotic agents [100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin](α-MEM-1X). To avoid effects on cell growth or drug resistance mechanisms, all subculture was performed on the antibiotic-free flasks. Once confluency was reached, a single flask would be trypsinized as outlined above, into at least two flasks; one with antibiotic and one without. If after two days the newly subcultured flasks were free of infection, the previous antibiotic containing flask would be discarded. In the rare event that the antibiotic containing flask was required to salvage the cell line, a minimum of two passages were performed without antibiotic, before continuing with cell growth studies.

To protect against permanent loss of cell lines due to infection or lethal accident, aliquots of healthy, rapidly growing cells were frozen and stored in liquid nitrogen for later retrieval if necessary. These were prepared by harvesting confluent cells by trypsinization and placing them in a 50 ml centrifuge tube. A 100 μl sample was drawn from this for determination of cell number using a haemocytometer and light microscope. The cell suspension was centrifuged (Beckman GPR Table Top) for 10 min at 1500 rpm. Supernatant was aspirated and cells were resuspended in an appropriate volume of α-MEM-1X + 40% FBS to yield a $2\text{-}4x10^6$ cells per ml suspension. An equal volume of α-MEM-1X + 20% DMSO was then added dropwise

with swirling, to yield a final suspension of 1-2x10^6 cells per ml in α-MEM-1X + 20% FBS + 10% DMSO. One ml aliquots were dispensed into 1.8 ml sterile cryovials which were placed in a -20°C freezer for two hours followed by overnight storage at -70°C. The following day cryovials were transfered to liquid nitrogen storage containers. To assess for viability of the frozen cells, one cryovial of each line was thawed several days after the freezing procedure and diluted into a T25 flask and cultured briefly.

5.3.2 Growth Curve

For each cell line, the normal rate of growth in the absence of antineoplastics or GST inhibitors was assessed. Healthy, rapidly growing cells from each line were harvested by trypsinization and counted in a Coulter Counter (Model ZM with Sampling Stand II). Appropriate dilutions were prepared and cells were seeded in triplicate at both 1x10^5 and 3x10^5 cells per 35 mm diameter well, in 6 well tissue culture plates (Corning). For each cell line, the cells from three culture wells were harvested every 24 hours for 5 days and counted in the Coulter Counter in triplicate. Mathematical manipulation of the raw data was performed on a Microsoft Excel 5.0 spreadsheet on an Apple Power Macintosh 6100/60 computer. To determine doubling time of the cell lines, linear regression was performed and the equation of the line was determined. Line equations were solved using Math-o-matic© demonstration version software on an IBM-compatible personal computer.

5.3.3 Cytotoxicity Studies

Cellular growth in the presence or absence of antineoplastic agents and GST inhibitors was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] Microculture Tetrazolium Assay, based on the protocol of

Alley et al (1988). Briefly, the MTT tetrazolium compound is taken up readily by cells and is reduced to the formazan product by mitochondrial succinate dehydrogenase and NAD(P)H-dependent microsomal enzymes (Berridge and Tan, 1993; Berridge et al., 1996). After a set incubation period, the culture medium-insoluble formazan crystals are solublized in DMSO. As the extent of metabolism to formazan ocurring should be related to the number of actively respiring mitochondria, spectrophotometric quantitation of the colorimetric density of the DMSO solution should be an indicator of the number of viable cells.

The standard assay procedure was as follows: Rapidly growing cells were harvested by trypsinization, counted with a haemocytometer (la fontaine NEUBAUER improved Bright Line) and innoculated at the appropriate concentration (10,000 cells per well for HT-29, SW620 and LoVo, 20,000 cells per well for Caco-2 as determined by density-time course assay in Figure 4, Section 6.1.2.1) in 96 well flat bottom microculture plates (Nunc), using a 12 channel pipetter (Brinkmann 12-transferpette 50-200 µl). Table 2 illustrates the standard plate setup for MTT assay. After 24 hours, half of the volume of each well was removed (100 µl) and replaced with 50 µl of GST inhibitor (at 3-fold concentration, prepared from fresh 100 µM stock in anhydrous ethanol), or medium containing the appropriate vehicle. Following 4 hours of incubation for Terrapin inhibitors or, 1 hour for ethacrynic acid, 50 µl of antineoplastic (at 4-fold concentration) or control medium was added to the wells, which were incubated for a further 4 hours. At this time 175 µl was removed from each well and replaced with GST inhibitor or control medium at working concentration. Plates were then incubated for 90-100 hours to allow sufficient time for cell death to occur. To

complete the assay, MTT (Sigma) at 5 mg/ml in PBS was diluted 1 in 5 with culture medium and 50 µl was added to each well. After 4 hours of incubation, 200 µl was removed from each well and replaced with 150 µl DMSO to solubilize the formazan crystals produced by mitochondrial/microsomal metabolism of the tetrazolium salt. To ensure thorough mixing, plates were placed on an orbital shaker (Bellco Glass Inc, Vineland NJ) set at 300 rpm for 5 min. Absorbance at 540 nm was measured on a Labsystems Multiskan LS microplate reader. Data were collected using Delta Soft 3 software on a Macintosh Classic II computer and electronically transferred to an Excel spreadsheet for manipulation. In certain experiments, the timing of GST inhibitor and antineoplastic additions was altered. These modifications are noted in the results section.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PB	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med
B	PB	Med	Bl	Con	Ex1	Ex2	Ex3	Ex4	Ex5	Ex6	Ex7	Med
C	PB	Med	Bl	Con	Ex1	Ex2	Ex3	Ex4	Ex5	Ex6	Ex7	Med
D	PB	Med	Bl	Con	Ex1	Ex2	Ex3	Ex4	Ex5	Ex6	Ex7	Med
E	PB	Med	Bl	Con	Ex1	Ex2	Ex3	Ex4	Ex5	Ex6	Ex7	Med
F	PB	Med	Bl	Con	Ex1	Ex2	Ex3	Ex4	Ex5	Ex6	Ex7	Med
G	PB	Med	Bl	Con	Ex1	Ex2	Ex3	Ex4	Ex5	Ex6	Ex7	Med
H	PB	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med	Med

Table 2: Standard setup for 96-well microculture plate assay

Legend: PB-Plate blank; Med-medium only, not used for assay; Bl-experiment blank containing medium only; Con- control for experiment, contains cells but no experimental compounds; Ex#-wells used for experiment, contain cells and test compounds or appropriate vehicle. All non-peripheral wells received identical work-up in terms of medium removal and replacement and addition of MTT. For a typical assay, the plate blank (PB) gave essentially zero absorbence. The media blanks (Bl) in lane 3 (rows B to G) were averaged and this value, the absorbence of the medium alone, was subtracted from all other lanes. The value remaining in lane 4, the control lane, after these subtractions represents the absorbence for colon cells unaffected by drug treatment and is assigned the value of 100%, to which all other drug treatment lanes are compared (e.g. an optical density reading in lane 8 which is 50% of lane 4 (after subtraction of lane 3 from both lanes) represents 50% survival.

5.3.4 Clonogenic Assays

To verify the cell proliferation results obtained under the MTT assay, a limited number of colony forming assays were performed on the HT-29 cell line. A single T80 flask of rapidly growing HT-29 cells was harvested by trypsinization and counted with a haemocytometer. Cells were seeded in T25 flasks at $1x10^5$ cells per flask in 5 ml of culture medium. One flask was prepared for each concentration of drug and/or inhibitor being evaluated, plus appropriate controls. Flasks were then incubated for 48 hours to place cells in exponential growth. To commence the assay, all medium was aspirated and replaced with 5 ml of either drug containing or control medium and incubated for the appropriate period of time for the type of assay and drugs involved. At the end of the drug exposure period, all medium was aspirated and each flask was washed with 10 ml of warm PBS. Cells were then harvested by addition of 750 µl of sterile trypsin and 10 min of incubation at 37°C. Eight mls of medium were added to each flask to halt trypsinization and cells were transferred to a 15 ml sterile Falcon tube. This process was performed in staggered batches of 3 to 5 flasks to minimize variation in exposure time to trypsin. Determination of cell number per tube was performed with a Coulter Counter (Model ZM with Sampling Stand II, Coulter Electronics, Burlington, ON). Two hundred µl of cell suspension were combined with 9.8 ml of Isoton II (Coulter Electronics, Burlington, ON) and counted in duplicate. Raw data from the Coulter Counter were entered into a Microsoft Excel 5.0 spreadsheet, on an Apple Power Macintosh 6100/60, for determination of the volumes of cell suspension and medium required to achieve the desired innoculation densities of 10^2 to 10^5 cells per well. Serial dilutions were performed in 15 ml sterile Falcon tubes. Two ml aliquots of suspension were dispensed using 5 ml serological

pipettes in 6-well flat-bottom culture plates (Corning, Costar Corporation, Oneonta, N.Y.). Three to six wells per innoculation density, per exprimental condition, were cultured for 14 days. After 7 days of culture, medium was removed from all plates and replaced with fresh medium. At the end of the incubation period, medium was again removed and replaced with 3 to 5 mls of methylene blue stain [0.5% (w/v) methylene blue, 50% (v/v) methanol, 50% (v/v) dH_2O]. Colony staining / fixing proceeded for approximately 25 minutes, after which stain was removed by aspiration and the 6-well plates were gently rinsed in a basin of tap water. Plates were drained and allowed to dry overnight. Counting of colonies (> 40 cells) was perfomed visually with the aid of a hand held counter. Counted colonies were marked individually with a red felt tip marker to ensure all were included and none was counted more than once. Data were entered into a Microsoft Excel 5.0 spreadsheet for determination of both average and relative survival, under each experimental condition.

5.4 Sample Preparation

5.4.1 Cytosol

To grow the large number of cells required for a cytosol preparation, several T80 flasks (4-6) were trypsinized and combined into a single sterile container. Sufficient α-MEM was added to allow dispensing of 90-100 ml of cell suspension into each of four to six 600 cm^2 square culture plates (Nunc). Cells were allowed to grow for 2 to 4 days, until an 80-90% confluency state was achieved. At this time harvest was performed. Feeding was generally not required due to the short growth period.

At harvest, all medium was aspirated and each square plate was rinsed with 30 ml of warm PBS. Ten ml of warm nonsterile trypsin was added to each plate and incubated for 10 min at 37°C. Twenty ml of cold α-MEM + 10% FBS was added to halt

trypsinization and cells were transferred to a 50 ml centrifuge tube (Falcon) pre-cooled on ice. A further 20 ml of cold PBS was used to rinse each plate, which was then added to the cell suspension in the centrifuge tube, to give a final volume of 50 ml. Cells were pelleted by a 10 min spin at 1200 rpm in a pre-cooled table-top centrifuge (Beckman). Supernatant was aspirated and cells were resuspended in 20 ml of ice-cold PBS. For large cytosol preparations, the cell suspensions of two 50 ml tubes were combined at this point and kept as such for the duration of the procedure. Cells were again pelleted by low speed centrifugation and all PBS was removed by aspiration. To facilitate cell lysis, 3 to 4 ml of ice-cold hypotonic HED (25 mM Hepes / 1.5 mM EDTA / 1 mM DTT, pH 7.4) buffer was added to the cell pellet. Following resuspension, cells were kept on ice and allowed to swell for 15 min. Homogenization was performed with a poyltron PT 7 tip at setting 8. Typically 2 to 4 bursts of 20 to 30 seconds were required for 90% cell breakage. Extent of cell lysis was monitored under a light microscope. An equal volume (3 to 4 ml) of ice-cold HED2G (25 mM Hepes / 1.5 mM EDTA / 1 mM DTT / 20% glycerol, pH 7.4) was added to the cell suspension and the entire lysate was transferred to a precooled Beckman screw-cap ultracentrifuge tube. All tubes were balanced and placed into a 70 Ti rotor. Ultracentrifugation was performed for 70 min at 106,000 g (40,000 rpm) in a Beckman L-80 Ultra to separate the membrane components from the cytosolic fraction. The supernatant was removed with a glass Pasteur pipette and transferred to a precooled test tube, total volume approximately 5.5 ml. The upper lipid layer was discarded and the membrane pellet was saved for further homogenization. Cytosol was aliquoted into 500 µl portions and dispensed into 1.8 ml cryovials and snap

frozen in liquid nitrogen. Samples were stored at -70 °C. Triplicate 5 µl aliquots of cytosol were saved for protein assay.

5.4.2 Membrane Pellet

The 106,000 *g* membrane pellet was suspended in 2 ml of storage buffer (10 mM Tris/20% glycerol/1 mM EDTA/pH 7.4) and transferred to a Wheaton Safe-Grind Potter-Elvehjem tissue grinder kit (Wheaton Inc., Millville, N.J.). Homogenization was performed using 8 to 10 slow up and down strokes. Membrane homogenate was aliquoted into 500 µl portions, dispensed into 1.8 ml cryovials, and snap frozen in liquid nitrogen. Samples were stored at -70 °C. Triplicate 5 µl aliquots of the samples were saved for protein assay.

5.4.3 Protein Analysis

Quantification of solubilized protein from cytosolic and membrane pellet samples was performed by the method of Bradford (1976). Bovine serum albumin (BSA) standard (1 mg/ml in water) was prepared in 250 µl aliquots and frozen for later use. Bio-Rad protein reagent was prepared from concentrate with 4 volumes of water, filtered, and placed in a bottle with a Brinkmann Dispensette (Brinkman Instruments Inc., Westbury NY) repeating dispenser. For each protein determination, one tube of BSA was thawed and 0, 10, 20, 30, 40, and 50 µg of protein were diluted in 2.5 ml of the diluted Bio-Rad reagent in 13x100 mm test tubes, vortexed and allowed to stand for 15 min. Triplicate 5, 10 or 20 µl aliquots of sample were diluted in an equal volume of reagent, vortexed and also allowed to stand. When the sample colour was visibly darker than the 50 µg standard, a second volume of Bio-Rad reagent was added to ensure absorbance readings would lie between the 0 and 50

μg range. The absorbance of the standards was used to generate a curve against which the cytosolic or membrane samples were compared. Absorbance was measured at 595 nm (with 465 nm as background reference) in regular size, disposable, polystyrene cuvettes, using a Beckman DU-65 spectrophotometer, with the first standard (0 μg protein in Bio-Rad reagent) as a calibration blank. The Quant II Quadratic Soft-Pac module (Beckman) was used to fit the standard curve via non-linear regression.

5.4.4 RNA Isolation

During the RNA isolation process, extreme caution was taken to protect samples against RNase contamination. Gloves were always worn and chemicals dedicated for RNA work were never used for other purposes. A complete set of pipettes and a gel electrophoresis apparatus were reserved for RNA work. All glassware, spatulas, Pasteur pipettes, bottles and cylinders were baked in an oven at 180 °C for at least 8 hours. All plasticware was sterile and disposable. All stir bars, plastic bottle lids, microfuge tubes, and pipette tips were autoclaved and reserved for RNA work. Water for preparation of all solutions was treated with diethylpyrocarbonate (DEPC) (0.1% (v/v)) and autoclaved. Subconfluent cultures of cells grown in P-100 tissue culture dishes (Falcon) were lysed directly in the culture dish by the addition of 1.5 ml of Tri-Reagent (Molecular Research Center Inc.). Total RNA was isolated using the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The cell lysate was passed several times through a pipette. After five minutes at room temperature, 0.3 ml of chloroform was added to the lysate, and the sample was vortexed for 15 seconds and allowed to stand at room temperature for 3 minutes. The resulting mixture was then centrifuged

at 12,000 x g for 15 minutes at 4 °C in a Beckman GS-15R Centrifuge (Beckman Instruments Inc.) to separate the mixture into a lower phenol-chloroform phase, an interphase and an upper aqueous phase. The upper aqeous phase was then removed and transferred to a fresh microfuge tube and the RNA precipitated by mixing with 0.75 ml of isopropanol. After 10 minutes at room temperature, the sample was centrifuged at 12,000 x g for 10 minutes at 4°C. After removal of isopropanol, the RNA pellet was washed once with 1.5 ml 75% ethanol, the pellet was air dried for 5 minutes in the fume hood and resuspended in 50 µl of DEPC water by incubating the solution for 10 minutes at 60 °C in a Dry Bath Incubator (Fischer Scientific Co.) heating block. RNA yield and purity was assessed by determining the absorbance at 260 and 280 nm using a Beckman DU-65 spectrophotometer. RNA yield is based on 1 OD_{260}= 40 µg RNA/ml and pure RNA should have an OD_{260}/OD_{280} ratio of 1.7 to 2.0. RNA was aliquoted and stored at -20 °C for subsequent electrophoresis and northern blot analysis.

5.5 Catalytic Activity of GSTs

5.5.1 Total GST Activity (CDNB Conjugation)

The change in optical density (ΔOD) associated with the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH was used as an indicator of total cytosolic GST conjugative activity (Habig et al., 1974). Samples of frozen cytosol were retrieved from -70°C freezer, thawed with warm water and then kept on ice. Based on the protein concentration determined at the time of preparation, cytosol samples were diluted with HEGD buffer (25 mM HEPES / 1.5 mM EDTA / 10% glycerol / 1 mM DTT, pH 7.4) to an end concentration of 1 mg/ml for use in this assay. Triplicate 20 µl

aliquots of this diluted cytosol were saved for later protein determination by the method of Bradford (Section 5.4.3).

All reagents were prepared fresh before beginning each assay. In a pre-cooled 13x100 mm glass test tube, the following reagents were mixed: 0.733 ml 0.1 M potassium phosphate buffer pH 6.5; 33.3 µl 60 mM CDNB (in ethanol); 200 µl cytosol (at 1 mg protein/ml), and 33.3 µl 60 mM GSH (reduced), added last to initiate the reaction. The final reaction volume was 1 ml and had the following components: 0.2 mg/ml cytosolic protein; 2 mM CDNB (in ethanol); 2.0 mM GSH and 0.1 M potassium phosphate, pH 6.5. After addition of the GSH, the test tube was vortexed for 3 seconds and the contents were transferred immediately, by pouring, to a disposable semi-microcuvette and placed in a Beckman DU-65 spectrophotomter. Temperature was held at a constant 25°C in the cuvette chambers with a Beckman temperature controller. Changes in OD were measured at λ=340 nm over a 5 minute period at 15 second intervals. For all reactions, water was used in the first cell position as a calibration standard. The absorbance readings were graphically displayed by the spectrophotometer at the end of each reaction. Based on this output, a time range was selected that represented the linear phase of conjugative activity. Using the Kinetics Soft-Pac module (Beckman) the ΔOD per minute was determined for each reaction occuring in cells 2 to 6 of the spectrophotometer. Non-enzymatic conjugation of GSH with CDNB was measured as the ΔOD associated with the complete reaction conducted without cytosolic protein (0.2 ml HEGD buffer substituted for cytosol). This blank reaction was run in triplicate, once for every set of experimental conditions. The ΔOD for this non-enzymatic reaction was subtracted from all reactions containing

cytosol so as to determine the contribution of GST protein to the total amount of CDNB conjugation occurring. The corrected ΔOD per minute was divided by 0.0096 (the extinction coefficient, ε=9.6 mM^{-1} cm^{-1}) to yield activity in units of nmol/ml/min. This value was divided by the final protein concentration (mg/ml) to give a final activity in nmol/min/mg of protein.

5.5.2 GST Pi Selective Activity (Ethacrynic Acid Conjugation)

The production of the GSH conjugate of ethacrynic acid was monitored as a selective measure of GST Pi catalytic function (Habig et al., 1974). The experimental procedure was nearly identical to that for determining total GST activity, with the exceptions described as follows. The components of the reaction cuvette were: 0.2 mg/ml cytosolic protein (0.2 ml of 1 mg/ml cytosol); 0.2 mM EA (33.3 µl of 6.0 mM stock in ethanol); 0.25 mM GSH (33.3 µl of 7.5 mM stock); 0.1 M potassium phosphate buffer, pH 6.5 (0.7333 ml of stock). ΔOD was measured at λ=270 nm under UV light, which necessitates the use of non-disposable quartz semi-microcuvettes. The extinction coeffiecient for this reaction is ε=5.0 mM^{-1} cm^{-1}.

5.5.3 GST Mu Selective Activity (*t*PBO Conjugation)

GST Mu activity was measured as a function of *trans*-4-phenyl-3-buten-2-one (*t*PBO) conjugation over time (Habig et al., 1974). The constituents of the reaction cuvette were: 0.4 mg/ml cytosolic protein (0.2 ml of 2 mg/ml cytosol); 0.05 mM *t*PBO (33.3 µl of 1.5 mM stock in ethanol); 0.25 mM GSH (33.3 µl of 7.5 mM stock); 0.1 M potassium phosphate buffer, pH 6.5 (0.7333 ml of stock). ΔOD was measured at λ=290 nm under UV light, which necessitates the use of non-disposable quartz semi-microcuvettes. The extinction coefficient for this reaction is ε=24.8 mM^{-1} cm^{-1}. In this

reaction absorbance decreases over time as opposed to the increase observed in the previous reactions.

5.5.4 GST Alpha Selective activity (CHP peroxidation)

The non-selenium dependent GSH peroxidase activity postulated as a class-selective function of GST Alpha, was measured as the spectrophotometric changes observed by the disappearance of NADPH as it is converted to $NADP^+$. This reducing power is required in the reduction of GSSG to GSH following GST-catayzed peroxidase activity. Using the method of Lawrence and Burk (1976), the cuvette reaction mixture consisted of: 0.2 mg/ml cytosolic protein (0.2ml of 1 mg/ml cytosol); 1.5 mM cumene hydroperoxide (CHP) (33.3 µl of 45mM stock in ethanol); 1 mM GSH (33.3 µl of 30 mM stock); 0.1 mM NADPH (10 µl of 10 mM stock); and 0.3 U/ml GSSG reductase (100 µl of 3.0 U/ml stock). After a 5 minute incubation at room temperature, the reaction was initiated by the addition of CHP and the cuvettes were scanned for 5 minutes at λ=340 nm under visible light in disposable semi-microcuvettes. Absorbance decreases over time and the extinction coefficient for this reaction is ε=6.2 mM^{-1} cm^{-1}.

5.6 Western Blot Analysis

Cytosol samples of all lines were diluted down to a common 1 to 2 mg protein/ml in HEGD buffer (25 mM HEPES / 1.5 mM EDTA / 10% glycerol / 1 mM DTT, pH 7.4) so as to load nearly equal volumes of sample to the electrophoresis gel. Protein concentrations were verified by the method of Bradford (1976) following dilution in buffer. Cytosol was prepared for electrophoresis in the following buffer (known commonly as Sample Buffer): 0.0625 M Tris-HCl (pH 6.8); 10% (v/v) glycerol;

2% (w/v) sodium dodecyl sulfate (SDS); 5% (v/v) β-mercaptoethanol; and 0.001% (w/v) bromophenol blue. Cytosol was diluted 5-fold in sample buffer and heated to 100 °C in boiling water for 5 minutes to denature the proteins. Cytosolic proteins were resolved by molecular weight using SDS-PAGE, according to the method of Laemmli (1970). For mini-gels, electrophoresis was carried out in a Bio-Rad Mini-Protean II gel apparatus, attached to a Bio-Rad Model 1000/500 Power Supply. For full-size gels, the standard Bio-Rad Protean II system was employed. Discontinuous polyacrylamide gels, either 100 x 75 x 1 mm (mini) or 200 x 150 x 1.5 mm (full) of the following composition were employed: stacking gel 4% T, 2.7% C (where % T represents the total percentage [w/v] of acrylamide monomer and N,N'-methylenebisacrylamide [BIS] crosslinker, and % C represents the amount of BIS crosslinker expressed as a percentage of the sum of acrylamide monomer and BIS), 0.125 M Tris-HCl buffer (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.1% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED); resolving gel for GST- 12% T, 2.7% C; MRP 7.5% T, 2.7% C, 0.375 M Tris-HCl buffer (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.05% (v/v) TEMED. The electrophoresis running buffer consisted of 0.025 M Tris-HCl buffer (pH 8.3), 0.192 M glycine , and 0.1% (w/v) SDS. For mini-gels, electrophoretic separations were conducted at 200 V constant voltage for approximately 45 minutes. Full-size gels were electrophoresed overnight at 15 mA constant current. Onto each mini-gel 10 µl of SDS-PAGE standards (GST: pre-stained low range, MRP: unstained broad range) (Bio-Rad) were loaded in the first or second lane to determine the approximate molecular weight (MW) of the immunoreactive bands. For full-size gels 15 µl of SDS-PAGE standards (unstained low range) (Bio-Rad) diluted 1:5 in gel loading buffer

were loaded adjacent to the pre-stained markers, to provide more accurate estimation of molecular weight.

The seperated proteins were transfered electrophoretically to nitrocellulose sheets (Hybond ECL, Amersham) by the method of Towbin et al (1979). The transfer was carried out either in a Bio-Rad Mini Transblot electrophoretic Transfer cell or in a Bio-Rad standard Transblot Cell, attatched to a Bio-Rad Model 200/2.0 Constant Voltage Power Supply. Mini-gel transfer was conducted at 100 V constant voltage for 1 hour with pre-cooled transfer buffer and a Bio-Ice cooling unit. Large gels were transfered at 60 V constant voltage for 5 hours, also with pre-cooled buffer and a tap water flow cooling unit.

Following transfer, the nitrocellulose was stained to assess transfer efficiency and to visualize unstained molecular weight standards. Membranes were immersed in 0.2% Ponceau S stain with agitation for 10 min and then rinsed with distilled water. Position of the unstained standards was marked on the membrane with a soft lead pencil. For immunodetection of proteins immobilized on the nitrocellulose, non-specific sites were blocked by incubating membranes in 20 mM Tris/137 mM NaCl/0.1% (v/v) Tween-20, pH 7.6 (TNT) containing 5% (w/v) skim milk powder and 0.001% (w/v) Thimerosal (Blotto) overnight at 4°C with constant, gentle agitation on an orbital shaker (Bellco Glass Inc. Vineland, NJ). Membranes were washed in alternating distilled water and TNT the following day for approximately 15 min. Incubation with primary antibody was carried out at room temperature for 1 to 1.5 hours with gentle agitation on an orbital shaker. Primary antibody dilutions in Blotto were as follows: GST Pi 1:50,000, GST Alpha (Biotrin) 1:1000, GST Alpha (Tew) 1:500, GST Mu 1:500, MRPm6 1:20. At the end of the primary incubation period the

membranes were drained and washed over six 5 minute intervals of alternating TNT and distilled water. Goat-anti-rabbit Ig horseradish peroxidase (HRP) conjugated secondary antibody incubations were conducted in Blotto for 1 hour at room temperature with gentle agitation at the following dilutions: GST Pi 1:5,000, GST Alpha (Biotrin) 1:1000, GST Alpha (Tew) 1:1000, GST Mu 1:2000, Donkey-anti-mouse Ig HRP conjugate was used with MRPm6 at a 1:1000 dilution. At the end of the secondary incubation period the membranes were again drained and washed over six 5 minute intervals of alternating TNT and distilled water.

Enhanced Chemiluminescence was carried out by mixing equal volumes of Amersham ECL detection reagents 1 and 2 and immersing membranes in the mixture for approximately 1 minute with agitation. The membranes were then drained, wrapped in Saran Wrap and placed in a Fisher Biotech cassette (8 x 10 inch) [no intensifying screens] with Hyperfilm ECL (Amersham) or Kodak X-Omat™ AR (Eastman Kodak Company, Rochester, N.Y.) film for the appropriate time period. Exposed films were developed in a Kodak M35A X-omatic processor in the Department of Molecular & Medical Genetics, University of Toronto.

For relative quantitative analysis, films were scanned using an LKB 2222-020 Ultroscan XL Laser Densitometer (Pharmacia LKB Biotechnology. LKB Producter Ab. Bromma, Sweden).

5.7 Northern Blot Analysis

5.7.1 RNA electrophoresis and transfer

For electrophoretic analysis, RNA samples (range 4.2 to 6.2 mg/ml; A_{260}/A_{280}=1.6 to 1.7) were diluted in DEPC water to yield appropriate concentrations such that 5, 10, and 20 μg of RNA could be loaded onto the gel using approximately

equal volumes (25-30 µl). RNA samples were prepared by adding 4.5 µl of diluted RNA to 2.0 ul of a 5X solution of gel running buffer (1X = 20 mM MOPS / 8 mM sodium acetate / 1 mM EDTA, pH 7.0), 3.5 µl of formaldehyde, 10 µl of ethidium bromide (1mg/ml). Samples were incubated for 15 min at 65 C, then placed on ice and 2 µl of gel loading buffer [50% (v/v) glycerol / 1 mM EDTA, pH 8.0 / 0.25% (v/v) bromphenol blue / 0.25% (w/v) xylene cyanol FF] was added. In addition, 3.0 µl of RNA ladder (prepared and treated as for samples) was separated on each gel. A standard kit (Life Technologies) contained the RNA fragments: 9.5 kb, 7.5 kb, 4.4 kb, 2.4 kb, 1.4 kb and 0.24 kb. RNA was resolved by electrophoresis following the method of Sambrook et al (1989). Electrophoresis was carried out in a Bio-Rad Mini-Sub-cell connected to a Bio-Rad Model 200/2.0 Constant Voltage Power Supply. Agarose gels (6.3cm x 10 cm x 1.5 cm) of the following composition were employed: 1% (w/v) agarose; 20 mM 3-[N-morpholino]propane sulfonic acid (MOPS) / 8 mM sodium acetate / 1 mM EDTA, pH 7.0; 2.2 M formaldehyde. The electrophoresis running buffer was 20 mM MOPS / 8 mM sodium acetate / 1mM EDTA, pH 7.0. Electrophoretic separation was carried out at 60 V constant voltage for approximately 2 hours. RNA integrity was assessed by comparing the relative intensities of 28s and 18s rRNA as visualized by a Viber Lourmat Flourescent Table UV trans-illuminator (Interscience Inc. Markham, ON) on ethidium bromide stained gels.

Following electrophoresis the gel was transferred to a pipette tip box and rinsed several times in DEPC water. The gel was then soaked for 20 min in 0.05 N NaOH, rinsed and soaked for 45 min in 10X SSC (150 mM sodium citrate, pH 7.0 / 1.5 M NaCl). A capillary transfer was set up according to the method of Sambrook et al (1989) using a nylon membrane prepared in accordance with manufacturers

instructions (Genscreen Dupont-NEN) and allowed to proceed overnight using 10X SSC as the transfer buffer. The following day the apparatus was disassembled and transfer efficiency assessed with methylene blue stain (0.02% (w/v) methylene blue / 0.3 M sodium acetate, pH 5.5). A 3 minute staining period was followed by destain with 1X SSPE (150 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA, pH 7.4) for 10-15 min. The positions of 28s and 18s rRNA bands were marked with a soft lead pencil and RNA integrity was assessed. Positions of the RNA ladder were also marked. The membrane was baked at 80 °C for 2 hours to fix the RNA to the nylon membrane and then wrapped in aluminum foil and stored at room temperature until hybridization

5.7.2 5'-Endlabeling of oligonucleotide probe

To detect mRNA for the various GST transcripts, six oligonucleotide probes specific for the GST isozymes were prepared according to the method of Sambrook et al (1989). The probe sequences are as follows: GSTA1 (5'-AAG TTC TTG GCC TCC ATG ACT GCG T-3') [David Waxman, unpublished - nts 738-762 of cDNA of Tu and Qian (1986)], GSTA2 (5'-ATG TTC TTG ACC TCT ATG GCT GGT T-3') [David Waxman, unpublished - nts 738-762 of cDNA of Rhoads et al. (1987)], GST M1 (5' TCA GCC ACT GGC TTC TGT CA-3') [Waxman et al. (1992) - nts 138-157 of cDNA of DeJong et al. (1988)], GST M2 (5'-AAT CTG GGT CAT AGC AGA GTT T-3') [David Waxman, unpublished - section of exon 5 of cDNA of Vorachek et al. (1991)], GSTM3 (5'-GTC AGA GCT GTA ACA GAG CCT TAT C-3') [David Waxman, unpublished - nts 362-386 of cDNA of Campbell et al. (1990)], GSTP1 (5'-GTT CTG GGA CAG CAG GGT CT-3') [Waxman et al (1992) - nts 398-417 of cDNA of Board et al. (1989)], α-tubulin (5'-GAC ATC TTT GGG GAC CAC ATC ACC ACG-3') [Waxman (1991)- nts 39-66 of rat α-tubulin 3'-end cDNA clone pT25 of Ginzburg et al. (1981)]. 2 μl (10 pmol) of the probe was

combined with 2 µl of T4 PNK Buffer, 1.5 µl H_2O, 12.5 µl [γ^{32}P]ATP (25 pmol) and 2 µl T4 PNK (20 units) in a microfuge tube and incubated for 45 minutes at 37°C. The reaction was halted by addition of 0.8 µl of 0.5 M EDTA and 20 µl TE8 (10 mM Tris HCl/ 1 mM EDTA, pH 8.0), and the tube was placed on ice. Forty µl phenol / chloroform / isoamyl (25:24:1) was added and the tube was vortexed and then centrifuged at max speed in an Eppendorf Table top model for 5 minutes. The upper aqueous layer was then removed and transferred to a new microfuge tube. Twenty µl of TE8 was added to the first tube and the spin procedure was repeated with the upper aqueous layer being pooled with that from the previous spin. 120 µl of ice-cold ethanol was then added and precipitation was allowed to proceed for a minimum of one hour, although typically overnight. Following precipitation the tube was centrifuged for 30 minutes at max speed and the supernatant was removed. The pellet was resuspended in 100 µl of TE8 and loaded onto a Phamacia NAP-5 column (Sephadex G-25) for oligonucleotide purification. The oligonucleotide was eluted by gravity using 10 mM sodium phosphate, pH 6.8. The volume of eluate was recorded (1.5 ml) and a 30 µl aliquot was removed to count radioactivity in a liquid scintillation counter. Activity in cpm / fmol of probe was calculated.

5.7.3 Northern Blotting

Prehybridization was conducted for 1 to 2 hours under the reagent and temperature parameters listed in Table 3. The solution consisted of 50 mM TES (Tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid) / 0.1% sodiumpyrophosphate / 0.1 mg/ml yeast tRNA / 1 M NaCl, pH 7.4 / 1 % SDS / 10X Denhardts (0.2% polyvinylpyrollidone / 0.2 % Ficoll type 400 / 0.2 % BSA) / formamide 0-25%.

Approximately 0.5 pmol (5-10 μl) of labelled oligo probe was added to the prehybridization (5 ml) solution to give a final concentration of 0.1 nM, which equates to a specific activity of about 600, 000 cpm/ml. Hybridization was allowed to proceed for 12-16 hours. The membrane was then washed twice at 5 minute intervals in 2X SSC (30 mM sodium citrate / 0.3 M NaCl, pH 7.0) at room temperature and then twice at 30 min intervals with 2X SSC / 1 % SDS at the hybridization temperature. The membrane was drained thoroughly, wrapped in Saran wrap and placed in a Fisher autoradiography cassette with two intensifying screens and Kodak X-ray film. The cassette was placed in -80 °C freezer for 2-5 days. Exposed films were developed in a Kodak M35A X-omatic processor in the Department of Molecular & Medical Genetics, University of Toronto.

Oligonucleotide	Formamide (%)	Temperature (°C)
GSTA1	5	45
GSTA2	5	45
GSTM1	0	45
GSTM2	0	45
GSTM3	10	45
GSTP1	0	40
α-tubulin	20	45

Table 3: Formamide concentration and incubation hybridization temperature for Northern Blot analysis

For relative quantitative analysis, films were scanned using an LKB 2222-020 Ultroscan XL Laser Densitometer (Pharmacia LKB Biotechnology. LKB Producter Ab. Bromma, Sweden).

On completion of Northern blotting for a particular GST mRNA, the blot was stripped in boiling 0.5 % SDS, checked by autoradiography for residual oligonucleotide hybridization and sequentially re-probed with the remaining oligonucleotides. Expression of specific mRNA was normalized for α-tubulin mRNA expression.

5.8 Determination of P-glycoprotein Expression

Duplicate samples of exponentially growing cells (10^5 cells in 5 ml of medium per 15 ml Falcon tube) from each line were provided to the laboratory of Dr. David Hedley of the Ontario Cancer Institute / Princess Margaret Hospital for analysis of Pgp expression. The technique employed to perform this assessment involved quantitative immunofluorescence image cytometry (D. Hedley, unpublished). Briefly, cells were labeled with the Pgp selective MRK-16 antibody by placing aliquots of the cultured cells on glass slides and evenly dispersing them using a Shandon Cytospin II at 400 rpm for 5 minutes, set at low acceleration. In order to unmask the Pgp epitope, cells were first incubated for 30 minutes at room temperature in a humidified chamber in 50 µl of 0.2 units/ml neuraminidase (Sigma) [0.2 µl of 100 units/ml stock in 100 µl]. Slides were then rinsed three times in PBS at 5 minute intervals. Non-specific binding sites were blocked with 50 µl of goat gamma globulin (Jackson Immuno Research) [1 µl of 1.83 mg/ml stock in 100 µl] per slide for 30 minutes at room temperature, with a coverslip in place to prevent evaporation. Cells were labeled with 50 µl of CY5 conjugated MRK-16 antibody (Courtesy of Dr. T. Tsuruo, University of Tokyo) [4.2 µl of MRK-16 in 100 µl of 238 µg/ml stock CY5] for 30 minutes at room temperature in a darkened, humidified chamber with a coverslip in place.

Slides were then rinsed three times in PBS at 5 minute intervals. Slides were counter-stained with 25 µl of AMCA (7-amino-4-methylcoumarin-3-acetic acid, succinimidyl ester) (Molecular Probes Inc.) [1 mg/ml stock in 50 ml PBS] for 10 minutes at room temperature in the dark. Slides were covered with 9 parts glycerol, 1 part PBS, 0.216 grams sodium azide (Sigma) and 2.32 g DABCO (1,4-diazabicyclo-{2,2,2}octane) (Sigma). Slides were stored in the dark at 4 °C until assay.

Quantification of MRK-16 immunofluorescence was performed using an Olympus BX50 fluorescence microscope fitted with an intensified CCD camera (Hamamatsu), interfaced to a SAMBA 4000 image analysis system (IPI Image Products International) with a Matrax IM640 frame grabber board. A UPlanFl 40X objective lens was utilized. Using a 360-370 nm excitation and 420 nm barrier filter cube, AMCA staining was used to create a mask defining the entire cell. The same field was then viewed with a 620-660 nm excitation and 660 nm barrier filter cube to measure antibody binding. Before analyzing slides, the black level was used to set the offset of the intensified CCD camera so that every pixel had a value greater than zero. Images displayed in false colours were used to ensure that detected fluorescence was not saturating the camera. A cell-free area of the slide was captured and stored in the system's memory as background noise. A homogeneously bright field, slightly brighter than the brightest cell, was also captured. The stored background was subtracted from this homogeneously bright field and the result stored in the system's memory. When a field was then selected for analysis, the program subtracted the stored background, divided the result by the homogeneous field minus the background and then multiplied the quotient by 255. This ensured that the maximum range of intensities between 0 and 255 was utilized. The cell image

obtained with the protein dye AMCA was used to create a mask of the cell, which was then overlaid on the CY5 fluorescence image, so that MRK-16 immunofluorescence was measured. Results for antibody labeling were expressed as the integrated fluorescence, which corresponds to the total amount of MRK-16 present. Frozen slides of CEM wt (wild type) and CEM 0.1 cells were used as controls, and colon cell samples were expressed as relative values.

5.9 Statistical Analysis

Data are expressed as means ± standard deviation (SD) throughout. Statistical analyses including one way analysis of variance (ANOVA), and Student-Newman-Keuls tests were performed using Instat 2.01 statistical software on a Power Macintosh 6100/60 computer.

5.10 Experimental Plan

1. Assay levels of GST Alpha, Mu and Pi protein and mRNA in four colon cell lines (Caco-2, HT-29, LoVo and SW620)

Cell culture was maintained and samples were prepared for assay as outlined specifically in sections: 5.4.1 (cell cytosol), 5.4.2 (membrane pellet) and 5.4.4 (RNA isolation). Western immunoblots and northern blots were conducted according to standard protocols as outlined in sections 5.6 and 5.7. Sample loading, polyclonal GST antibody concentrations and film exposure times were as outlined for individual experiments.

2. Determine total and class-selective catalytic activity of GSTs in these cell lines.

Total GST catalytic activity was determined as outlined in section 5.5.1. Class selective activities were determined as outlined in sections 5.5.2 (GST Pi selective conjugation), 5.5.3 (GST Mu selective conjugation), and 5.5.4 (GST Alpha peroxidase activity). Determination of effective inhibition of CDNB conjugation by Terrapin GST inhibitors was as follows: GST Pi selective inhibition was studied using CDNB conjugation assay (section 5.5.1) on HT-29 and Caco-2 cell cytosol, with 0.05 mM GSH and 50, 5.0, 0.5, 0.05 and 0.005 μM of TER 117. The contribution of GST Alpha to total activity was assessed by repeating the assay with the substitution of the Alpha inhibitor TER 143. Caco-2 and HT-29 cell lines were used in this assay to represent GST Alpha expressing and non-expressing colon cancers with similar amounts of GST Pi protein. The ability of the TER 117 diethyl ester

(TER 199) to enter intact cells and inhibit GST was assessed by exposure of cells in culture to 100, 10 and 1.0 μM of TER 199 for 4 hours, followed by rapid cytosol harvest and CDNB conjugation assay. Inhibition of GSH peroxidase activity was determined using the protocol of section 5.5.4 with the addition of 50, 5.0, 0.5 and 0.05 μM of TER 117, 143 and 206 in LoVo cytosol.

3. Determine the level of other resistance mechanisms (MRP and Pgp) in these cell lines.

MRP protein expression in the membrane pellet fraction was assessed by immunoblot, using the monoclonal anti-human MRPm6 antibody. Pgp expression was determined using quantitative immunofluoresence image cytometry with CY5 conjugated MRK-16 anti-Pgp antibody.

4. Determine the relative resistance of the four cell lines to Dox.

The MTT microculture colourimetric assay was initially verified as a valid means for determining cell growth/cytotoxicity in these colon cancer cell lines. Production of formazan in relation to cell number was validated, maximum culture time and cell number maintaining exponential growth was assessed and a time-course for loss of cellular respiration following Dox exposure was determined. IC_{50}s of Dox in these cell lines were assessed under chronic (96 hours) and acute (4 hours) exposure conditions, using one third-log serial dilutions (90, 30, 10, 3.3, 1.1, 0.37, 0.123 & .041 μM) of Dox. IC_{50}s of chlorambucil (Chlor) in these cell lines were assessed for

later use as a positive control for GST inhibition effects. Identical acute-exposure assay conditions were employed with serial dilutions of 270, 90, 30, 10 and 1.1 μM of Chlor.

5. Attempt sensitization of cell lines to Dox with isozyme selective inhibitors.

Toxicity of Terrapin GST inhibitors and ethacrynic acid (EA) were determined prior to GST inhibitor - Dox combination studies. For HT-29, SW620 and LoVo cell lines 10, 3.3 and 1.1 μM of either TER 199 / 183 were used in conjunction with Dox (0.041 to 90 μM) or Chlor (1.1 to 270 μM). For Caco-2 cells 30, 10 and 3.3 μM of Terrapin inhibitor were employed. EA was used consistently at 90, 30 and 10 μM for all cell lines and both antineoplastics.

Clonogenic assays were conducted to verify results of the MTT assays using HT-29 cells. Cytotoxicity was assessed using 0.041 to 10 μM of Dox or 1.1 to 90 μM Chlor. Combination studies were performed with 0.123 to 1.1 μM of Dox or 10 to 90 μM of Chlor in conjunction with 30 and 3.0 μM of TER 199.

6. Results

6.1 Cell Culture

6.1.1 Growth Properties

All cell lines grew in α-MEM supplemented with 10% FBS, with each line displaying characteristic and unique growth properties (Figure 2). Caco-2 cells were very density dependent and grew logarithmically only beyond a certain critical number. SW620 grew well as isolated colonies with LoVo and HT-29 lying in the middle of these extremes. Two interesting features of the LoVo cell line were: 1) its inability to maintain a high confluency culture, even with frequent changes of growth medium and; 2) a low plating efficiency, compared to the other cell lines (Figure 2). The doubling times of these lines were determined during the exponential growth phase and are as follows: SW620 - 32 hr; LoVo - 34 hr; HT-29 - 35 hr, Caco-2 - 39 hr.

6.1.2 MTT Assay

6.1.2.1 Initial workup

The MTT assay is an indirect measurement of cell growth. as it measures the ability of cells to metabolize dye in a population dependent manner, rather than counting the actual number of living cells (Alley et al., 1988). As such, a considerable amount of preparatory work must be performed to ensure the end optical density readings recorded in this assay, correlate with the number of cells present.

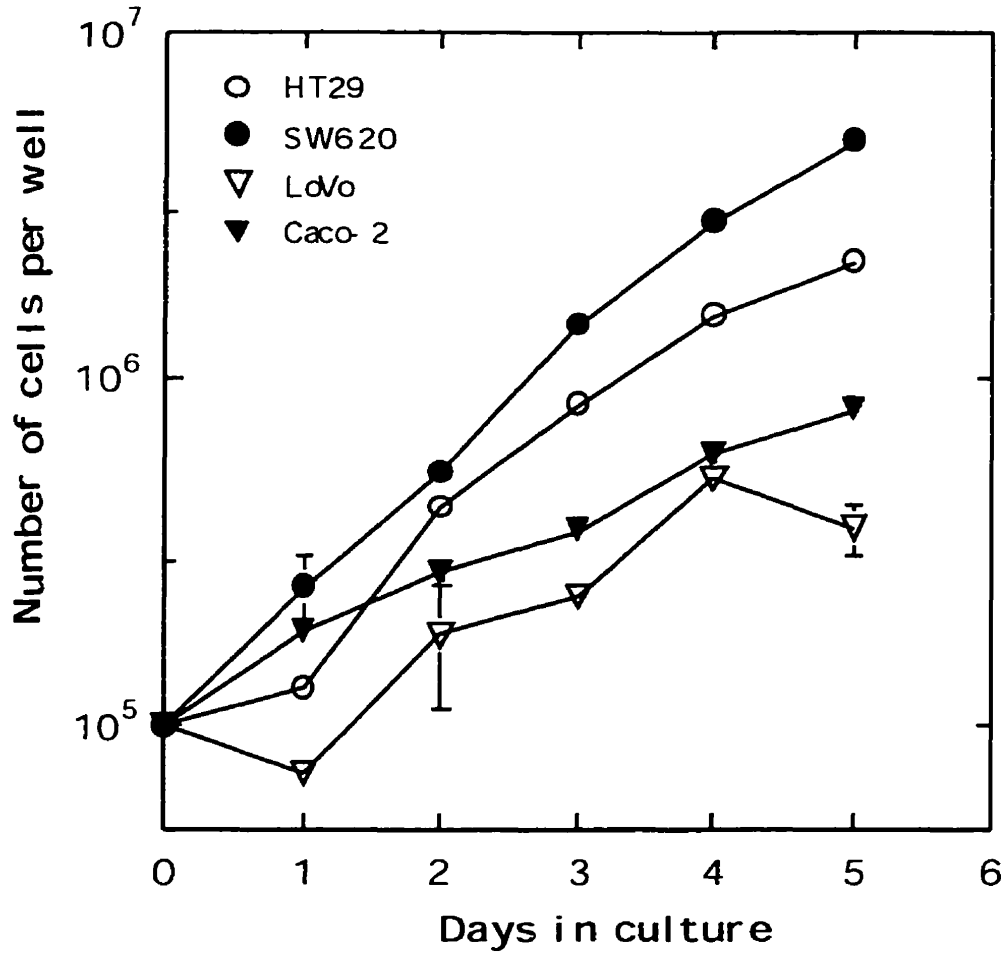

Figure 2: Growth curve of colon cancer cell lines

All cell lines were seeded at 10^5 cells per well in triplicate in a 6 well plate. For each line five plates were prepared and one plate was counted per day for 5 days. Each data point represents the mean ± SD of triplicate determinations.

The first preparatory experiment was the determination of whether the cells were able to metabolize the dye, and whether the extent of this metabolism was related to the cell number. To do this, three 96 well plates were prepared as shown in Table 2 (section 5.3.3), and placed in the cell culture incubator for 2 hours. This time frame was chosen as it is sufficiently long to allow the cells to adhere to the bottom of the plate, but not long enough to allow replication to begin. The remainder of the assay was conducted as outlined in section 5.3.3. Figure 3 illustrates that for HT-29 cells, insufficient dye is being metabolized below $1x10^5$ cells per well to be accurately read by the plate reader and that between $5x10^5$ and $5x10^6$ cells there is a linear

relationship between the number of cells in the well and the optical density reading. A similar correlation was observed with Caco-2, LoVo and SW620 cells (data not shown).

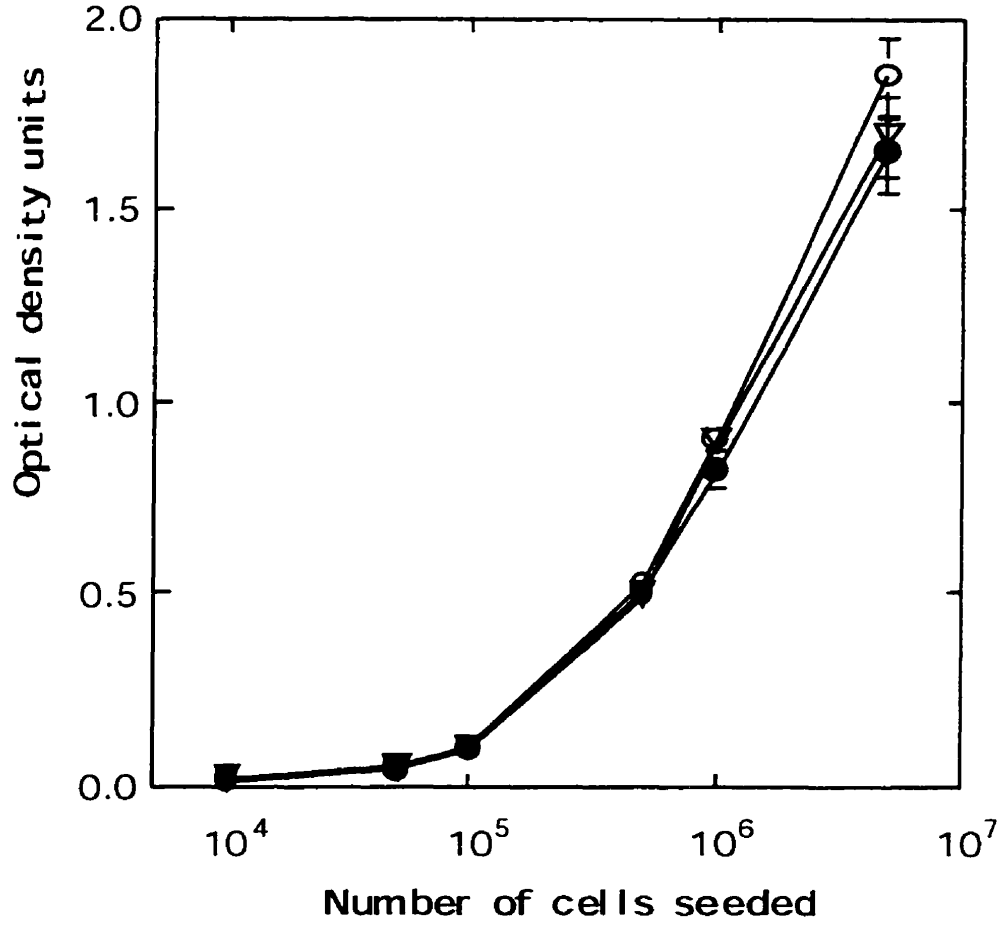

Figure 3: Determination of relationship between optical density and cell number in the MTT assay.

Known quantities of HT-29 cells were seeded into wells of triplicate plates and allowed to adhere for 2 hours. MTT dye was added and incubated for 4 hours and then developed as per standard protocol. Each point is the mean ± SD of sextuplicate determinations. Each line represents a single 96 well plate. Plates are displayed separately to demonstrate the minimal plate to plate variation that can be achieved in this assay.

Dox requires cellular replication to occur before its main mechanism of toxicity can take place (Hochauser and Harris, 1993). Mitochondria require time to stop actively respiring after the point of loss of cellular viability has been reached (Carmichael et al., 1987). For these reasons, it was necessary to determine the

maximum amount of time cells could be sustained in culture, without medium replacement. Additionally, due to the limited linear range of the assay (0.1 to 2.0 OD units), conditions had to be optimized such that the OD values for control wells would lie near the upper end of this range. This allows drug treatment wells to fall on the lower, but still readable, end of the spectrum. To determine these conditions, four densities of cells (1,000, 2,500, 5,000 and 10,000 cells per well) were seeded in quintuplicate 96 well plates and one plate was developed every 24 hours over a 120 hour period.

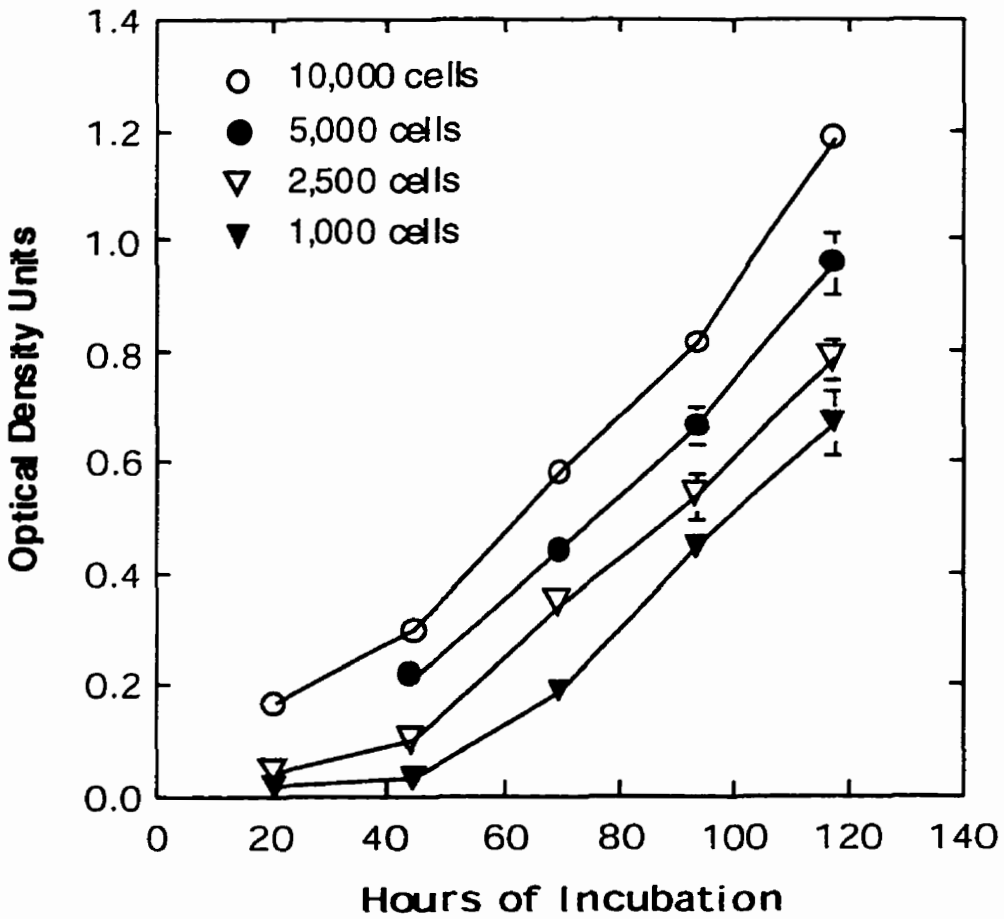

Figure 4: Assessment of cell growth in microculture over time

HT-29 cells were plated in quintuplicate plates at four densities (10,000, 5,000, 2,500 and 1,000 cells per well). One plate was developed at approximately 24 hour intervals over 120 hours. Each data point is the mean ± SD of sextuplicate determinations

Figure 4 illustrates the increasing OD readings associated with higher seeding density and the expansion of cell number over a 24 to 120 hour time frame. The essentially parallel, linear plots, indicate that for this line: 1) optical density is associated with cell number; 2) the rate of cell growth is independent of seeding density; and 3) the maximum OD reading that can be achieved is approximately 1.2 units. Similar assays with the remaining cell lines indicated that each one could be sustained for 90-100 hours and the maximum number of cells per well to be seeded was 10,000 for HT-29, SW620 and LoVo, while the Caco-2 line can be seeded at 20,000 cells per well (data not shown).

6.1.2.2 Dox Toxicity

The next preparatory experiment was the determination of how many hours of Dox incubation were required to generate an interpretable and reproducible dose response curve, in each of the cell lines. Using values from the previous steps, a series of experiments was performed to determine the optimum assay conditions. Figure 5 depicts a typical curve that shows the type of difference that can be observed by changing the Dox incubation period by just a single day. Since many of the experiments to be conducted beyond this point required comparison of the effects of identical assay conditions on the four cell lines in culture, a set of assay parameters had to be established that, while perhaps not ideal for each line, could be practically implemented and applied to all lines equally. It was decided that cells would be plated and then allowed to grow for 24 hours, following which Dox and/or GST inhibitor would be applied. The cells under assay would then be left undisturbed for 96 hours (120 hours total assay time) prior to the addition of MTT dye.

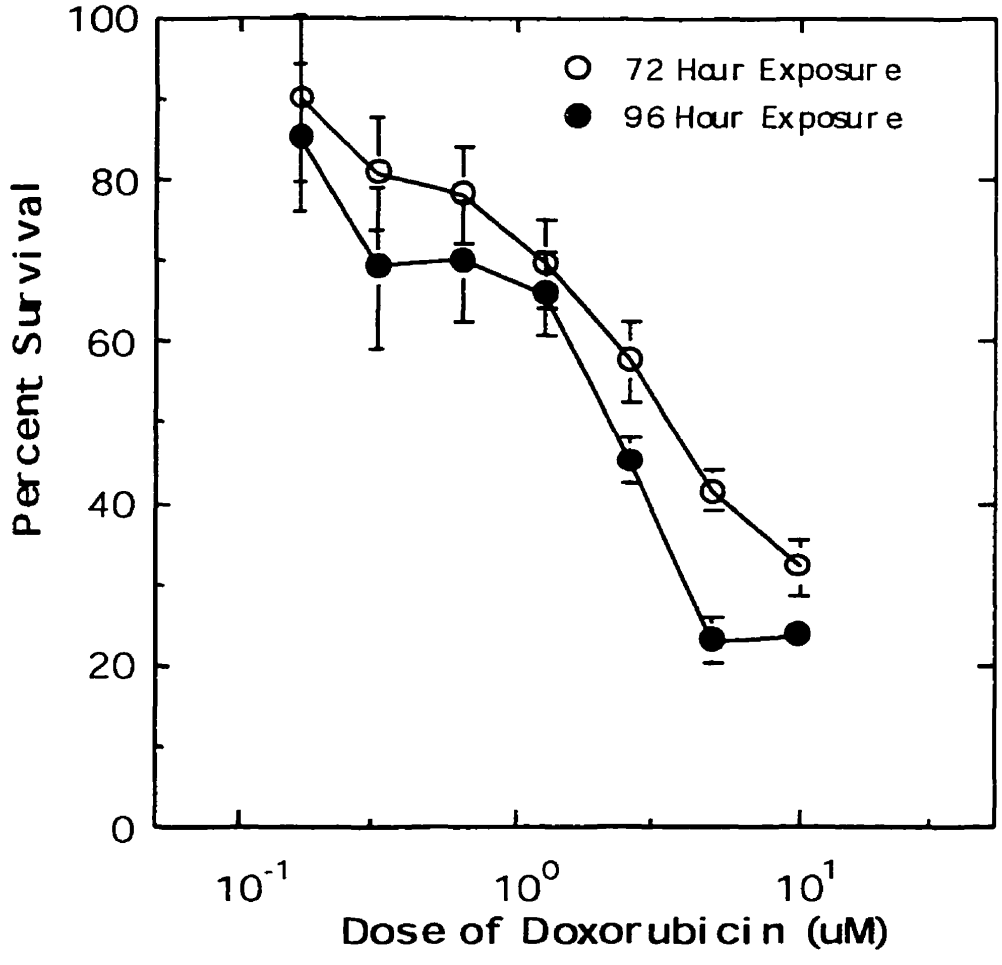

Figure 5: Effect of varying drug exposure time on cell survival

Four replicate, standard format plates were prepared using HT-29 cells and increasing doses of Dox. Two plates were developed on day 4 of the assay (72 hours of incubation) and 2 were developed on day 5 (96 hours). Each point is the mean ± SD of two plates of sextuplicate determinations (n=12).

The four cell lines used in this study are all derived from tumours of human patients diagnosed with adenocarcinoma of the colon. Of these lines, only Caco-2 is reported to be derived from a patient who received chemotherapy (cytoxan and 5-fluorouracil) (ATCC, 1992), however, details regarding the extent of therapy are not available. These lines were selected based on their broad range of sensitivities to Dox (and ability to metabolize MTT) alone, and are not intended to reflect stepwise degrees of resistance to Dox or other antineoplastics. Initial experiments on response to Dox exposure in these cell lines were conducted with Dox being left in the

culture medium for the duration of the 96 hour drug incubation period. This type of exposure has been termed "chronic." During the course of study on the ability of putative GST inhibitors to potentiate the toxicity of Dox, it was deemed necessary to alter the length of antineoplastic exposure to a much shorter period. These later experiments, referred to as "acute" exposure, were conducted with Dox in the cell culture medium for only 4 hours. At the end of this period, 175 µl of the 200 µl of culture medium was removed and replaced with fresh medium, without Dox.

The effect of this change in experimental procedure was most noticeably observed in the least sensitive cell line, Caco-2. Acute exposure shifted the IC_{50} of Dox in this line more than a full log unit to the right. IC_{50}s of Dox in HT-29 and LoVo were shifted approximately half a log and the most sensitive SW620 cells were affected the least. The rank order of sensitivities was unaltered. IC_{50} values are presented in Table 4.

As can be seen in Figure 6, there is a remarkable 1,000-fold range of sensitivities from the most sensitive SW620 cells to the most resistant Caco-2 cells, under acute exposure conditions. Figure 7 demonstrates the dose-response occurring as a result of the chronic-type exposure. To what extent resistance of the Caco-2 line can be related to previous chemotherapy with 5-FU is not known (ATCC, 1992).

Dose-response for all lines was consistent over the entire study period, which represents approximately 50 - 70 passages since first isolation from the primary tumour.

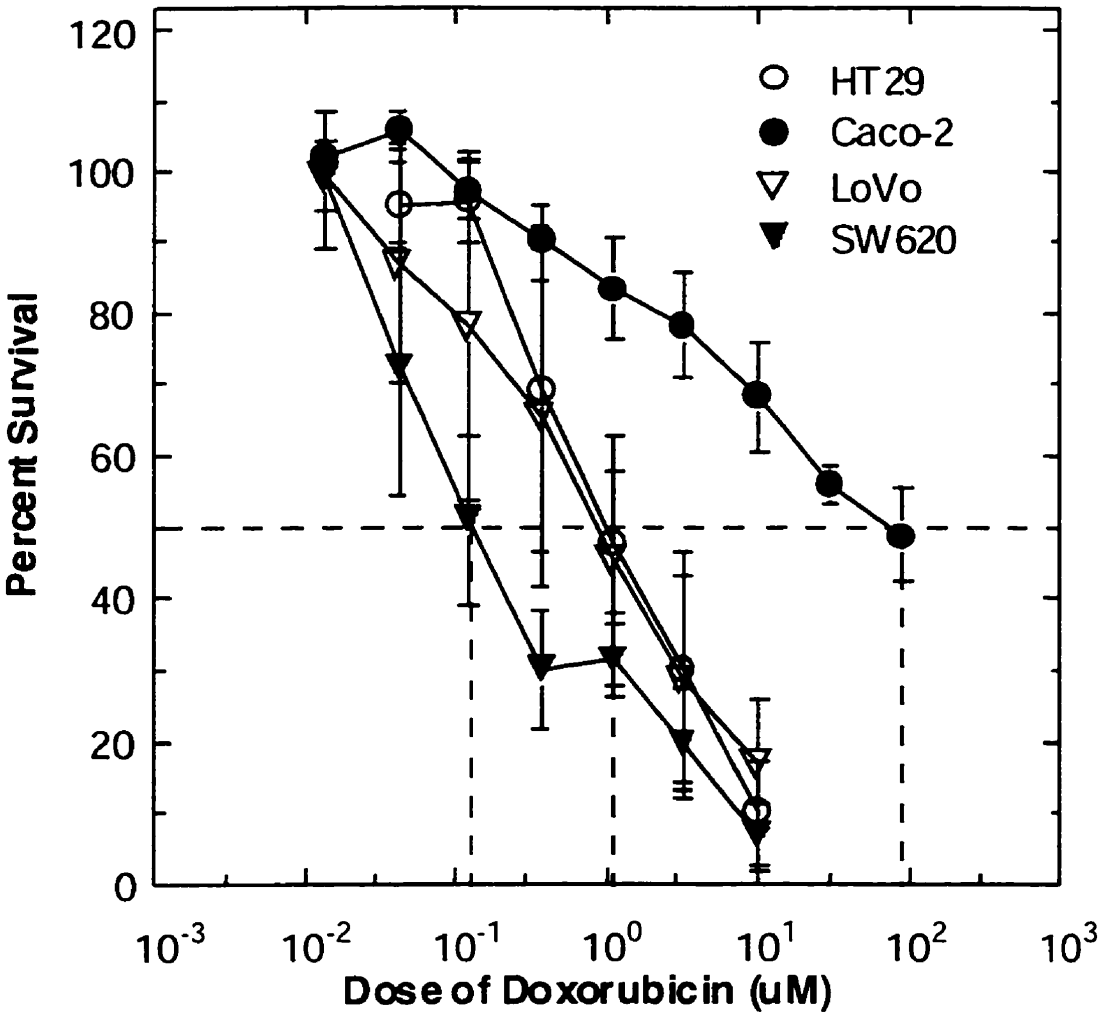

Figure 6: Comparison of sensitivities of four colon cancer cell lines to doxorubicin (acute exposure).

Results of multiple MTT assays over several months, were combined to produce these dose response curves. Each data point represents the mean ± SD of data produced from 3 to 6 separate experiments. Individual assays were composed of two 96 well plates each with sextuplicate determinations (Total: 36-72 data wells). The mean and SD of each experiment were combined to produce the final cell survival values. 100 percent survival is represented by the mean corrected optical density of the control lane (Con in Table 2). Dox exposure was limited to 4 hours, following which culture medium was removed and replaced with drug-free medium.

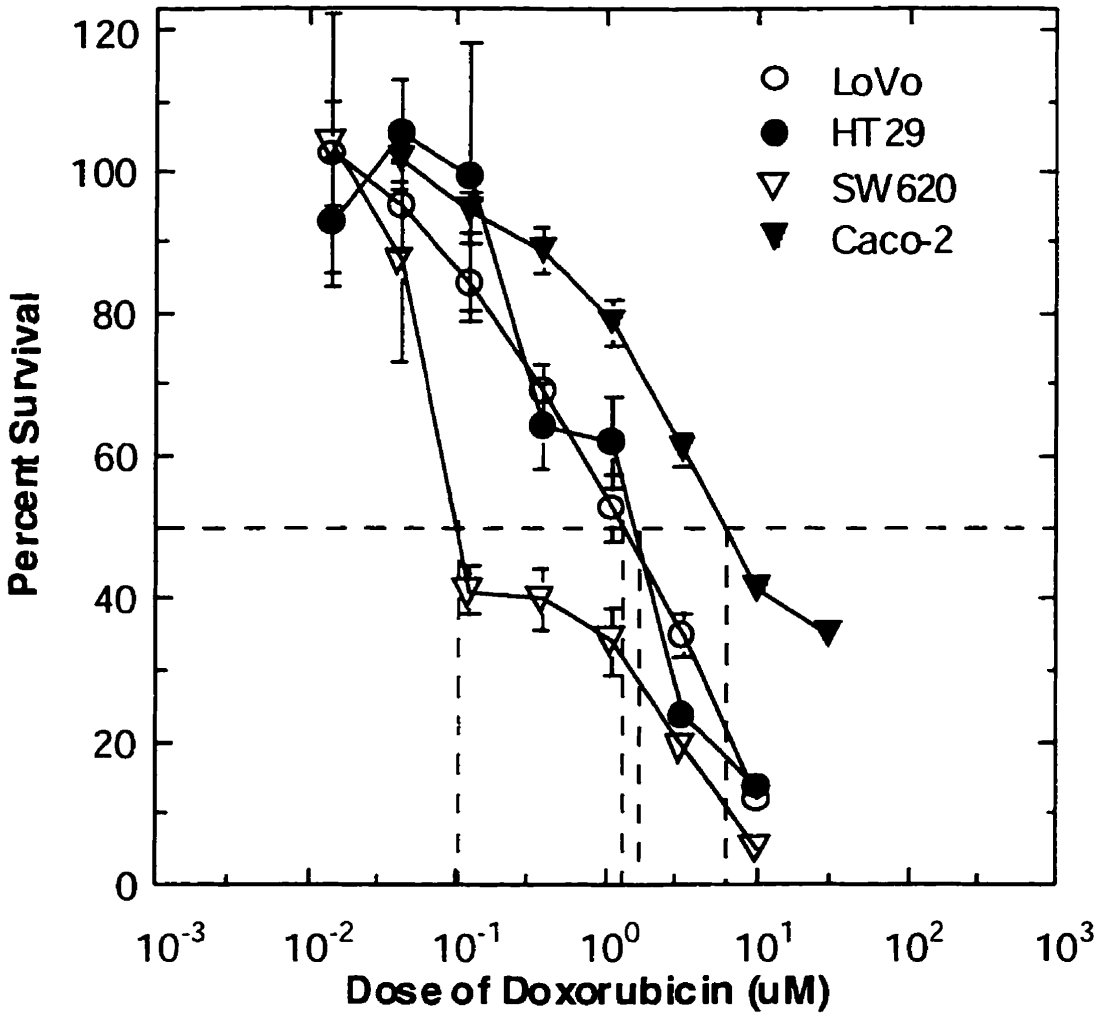

Figure 7: Comparison of sensitivities of four colon cancer cell lines to doxorubicin (chronic exposure).

Results of initial, chronic exposure MTT assays, were combined to produce these dose response curves. Each data point represents the mean ± SD of data produced from 1 to 3 separate experiments. Individual assays were composed of two 96 well plates each with sextuplicate determinations (Total: 12-36 data wells). The mean and SD of each experiment were combined to produce the final cell survival values. 100 percent survival is represented by the mean corrected optical density of the control lane (Con in Table 2). Dox was allowed to remain in the culture medium for the remainder of the assay.

6.1.2.3 Chlorambucil Toxicity

In addition to the MTT cytotoxicity assays performed with Dox on our colon cell lines, the resistance profile was also assessed for the alkylating agent chlorambucil (Chlor). The effects of this agent, used in conjunction with the Terrapin GST inhibitors, have recently been determined by other investigators (Morgan et al., 1996). There is now data to indicate that the cytotoxic effects of Chlor can be moderately potentiated by TER 199 in HT-29 cells. Hence, we conducted basic toxicity assays and combination studies with Chlor on HT-29 and other cell lines, with the intent of using them as positive controls. The first step then, as was conducted for Dox, was a determination of the IC_{50} of Chlor. Figure 8 depicts the dose-response relationship for SW620, Caco-2 and HT-29 (LoVo cells were not available for study at the time these assays were performed). This agent appears to be approximately 2 orders of magnitude less potent than Dox, and the range over which the IC_{50}s for the different cell lines (LoVo not determined) span is considerably less; 1 order of magnitude for Chlor, compared with 3 orders of magnitude for Dox. Most striking is the greatly reduced difference in the IC_{50}s of Caco-2 and HT-29 cells (145 μM vs 130 μM) which were previously separated nearly 100-fold with respect to Dox sensitivity.

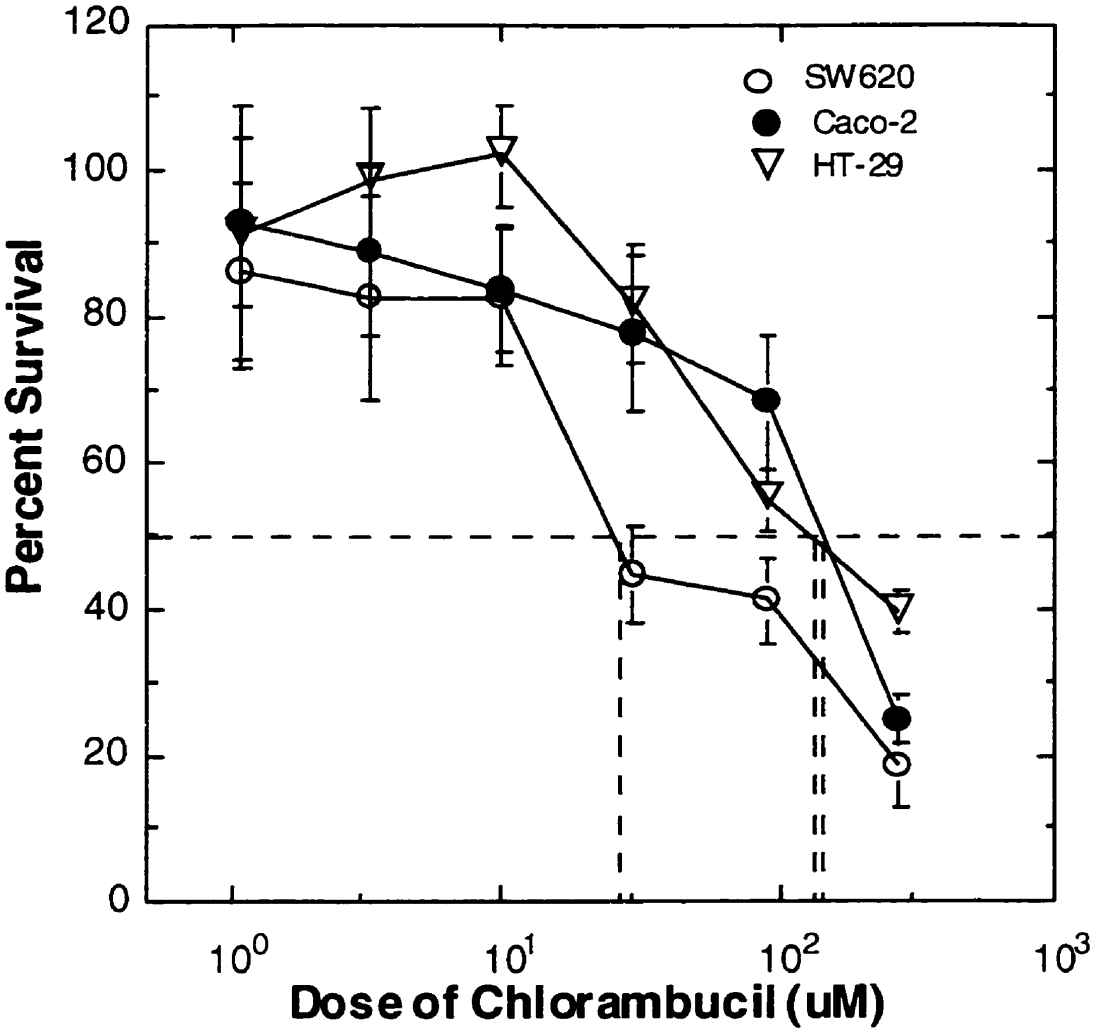

Figure 8: Comparison of sensitivities of three colon cancer cell lines to chlorambucil (acute exposure)

Results of a 4 hour Chlorambucil incubation on HT-29, Caco-2 and SW620 cells. Data points represent mean ± SD of sextuplicate determinations from duplicate 96-well plates in a single experiment (n=12). Data for the three cell lines are taken from independent experiments to generate this comparative graph.

6.1.2.4 GST Inhibitor Toxicity

A major goal of this work was to study the possible potentiation effects of a number of putative GST inhibitors on the cytotoxicity of antineoplastics, specifically the anthracycline Dox. As part of this project, the toxicity of these experimental compounds, in the absence of other cytotoxic substances, had to be assessed. To do this, the MTT assay was performed under the same conditions as the Dox dose-

response study, with the exception that it was conducted using the GST inhibitors alone. For all inhibitors, toxicity was assessed on a chronic exposure basis. Though many of the combination studies later employed much shorter drug incubations, this method provided a benchmark for the maximum toxicity that could be attributed directly to the inhibitors.

	Dox (μM)		Chlor (μM)
Cell Line	Acute	Chronic	
Caco-2	78	6.7	145
HT-29	1.0	1.5	130
LoVo	0.86	1.3	N.D.
SW620	0.13	0.1	25

Table 4: IC_{50}s of Dox and Chlor in colon cancer cell lines

Figure 9 displays the cytotoxicity caused by the Terrapin GST class-selective inhibitors (TER 183, 199, & 206) as well as that for EA on HT-29 cells. All three Terrapin compounds were fairly toxic to the colon cell lines. The IC_{50}s ranged from 8 to 40 μM (Table 5) and were independent of Dox sensitivity and cell doubling time. Shorter inhibitor exposures (4 to 8 hours) resulted in a lesser degree of toxicity, however the rank order of potencies of the compounds was unchanged (Data not shown).

	IC_{50} (μM)		
Cell Line	TER 183	TER 199	TER 206
SW620	10	26	20
LoVo	8	26	16
HT-29	13	20	20
Caco-2	20	28	40

Table 5: IC_{50}s of Terrapin GST inhibitors in colon cancer cell lines

Dose of Terrapin inhibitor causing 50% reduction in optical density compared to control. Values based on a single chronic exposure assay of sextuplicate determinations (n=6).

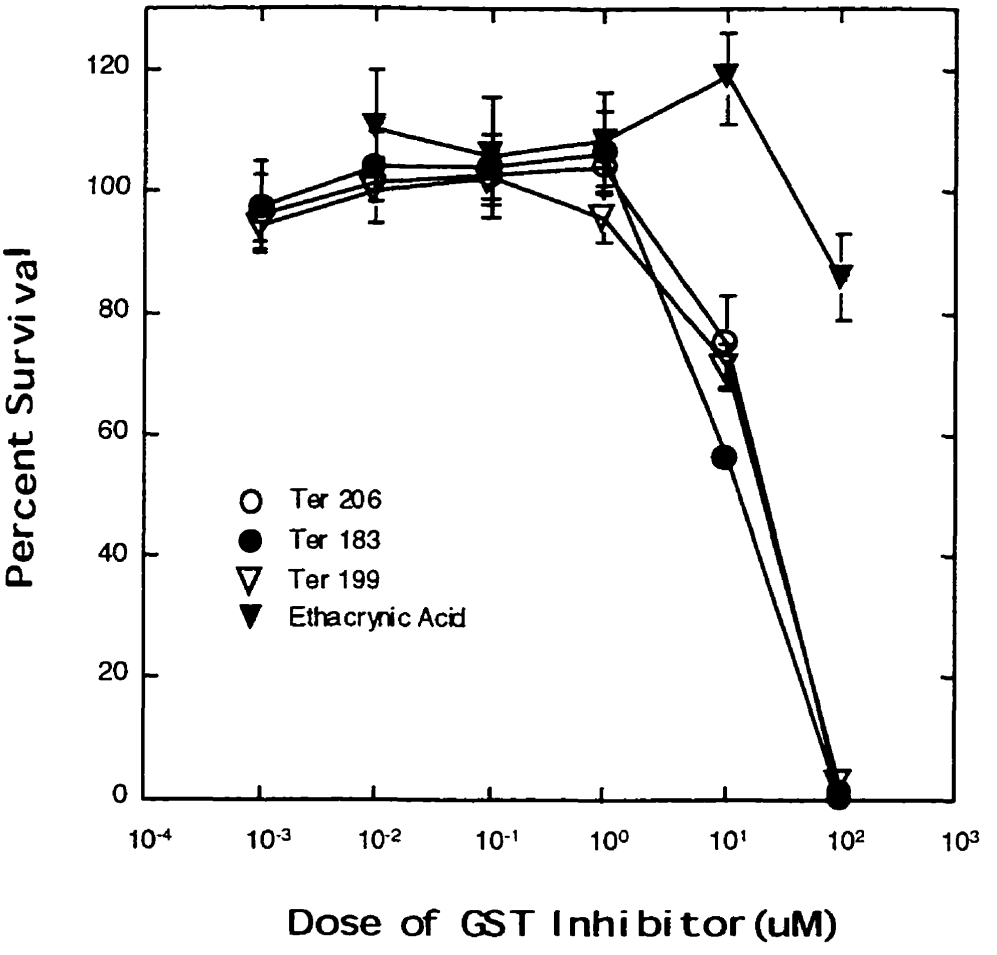

Figure 9: Toxicity of GST inhibitors to colon cancer cell lines

Data points are the mean ± SD of sextuplicate determinations from a single 96-well plate. For Terrapin compounds, three replicate plates of HT-29 cells were prepared and each one incubated with a single test compound (TER 183, 199 or 206). On a separate day, a single plate of HT-29 cells was prepared for assay with EA. All drug exposures were chronic. 100% represents OD of cells cultured in the absence of GST inhibitors.

6.1.2.5 Combination Studies

6.1.2.5.1 Ethacrynic Acid

As EA is the classic GST inhibitor (Tew et al., 1988) and also the resistance modulator currently being tested in clinical trials (O'Dwyer et al., 1991; Lacreta et al., 1994; Massaad et al., 1994), it was used for preliminary studies on which later experiments would be based. Additionally, EA was readily available from Sigma in

large quantities and thus was used in our pilot studies so that the Terrapin compounds could be preserved. For this and all other combination studies, a period of incubation with inhibitor alone (referred to as pre-incubation) was employed before the addition of Dox. Initially we evaluated 1, 2 and 4 hour pre-incubations, used in conjunction with a chronic (72 hr) Dox exposure. For this particular assay (Figure 10), EA remained in the culture medium along with Dox for the entire chronic incubation period.

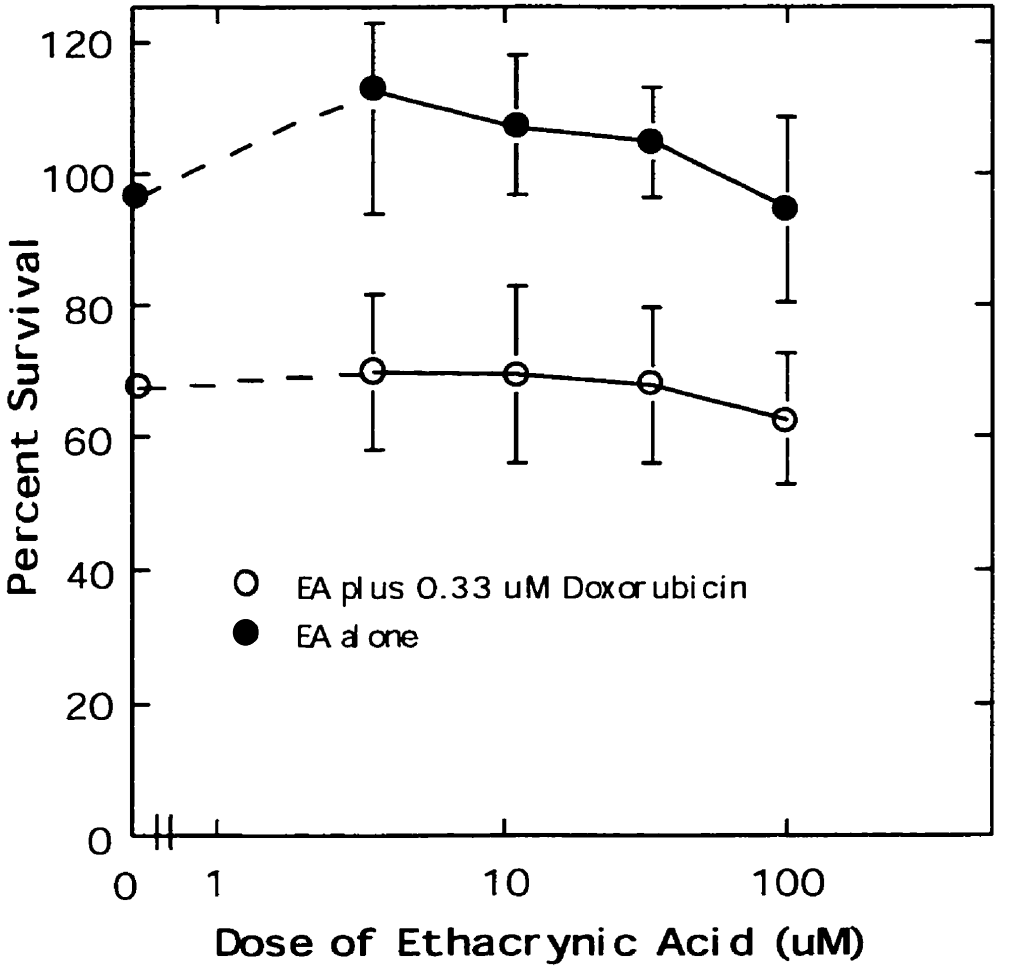

Figure 10: Preliminary Dox - EA combination study

A one hour pre-incubation of EA combined with a chronic exposure of Dox at 0.33 µM to HT-29 cells. Inhibitor remained in medium with antineoplastic for the entire incubation. Data points are the mean ± SD of duplicate plates, each of sextuplicate determinations (n=12). Dashed line projection to the y-axis represents toxicity in the absence of EA (ethanol control only).

As EA was known to be non-toxic at up to 100 μM, this was the maximum concentration assessed for combination studies. In addition, one third serial dilutions of EA were employed (33.0, 11.0 and 3.6 μM) in conjunction with a single dose of Dox. The dose of Dox that was chosen was that which could reliably produce near 50% toxicity to the individual line. For this particular assay 0.33 μM Dox was selected. As can be seen in Figure 10, EA was unable to potentiate the effects of Dox on HT-29 cells. The results of the 2 and 4 hour incubation were not different from the 1 hour incubation and hence are not included in the figure. Similar results were obtained with Caco-2, LoVo and SW620 (data not shown).

Subsequent to the failure of EA to effect potentiation under chronic exposure conditions, assay parameters were altered to more closely reflect those which may be occurring *in vivo (i.e.* EA pre-administration, Dox administration, Dox elimination and then post-chemotherapy EA maintenance dosing). To do this, the same 1 hr EA pre-incubation was employed in conjunction with a shortened 4 hr Dox exposure. Following this, the culture medium was removed and replaced with medium containing EA alone, at the same concentration as used during the pre-incubation period. This too, failed to result in potentiation (data not shown).

A third combination of Dox and EA was assessed, whereby both agents were removed at the end of the 4 hour Dox incubation and cells were cultured in the absence of any pharmacological agents. This also showed no potentiation (Figure 11).

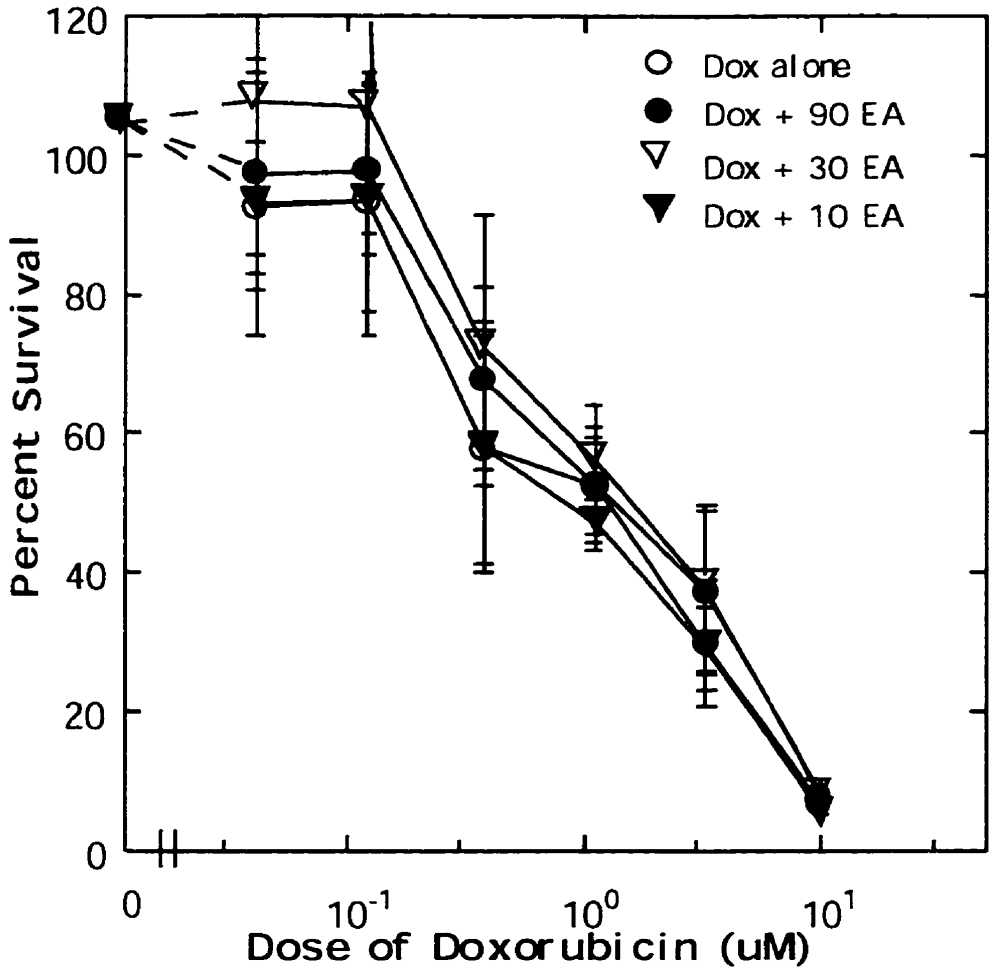

Figure 11: Assessment for potentiation of Dox cytotoxicty by ethacrynic acid

Eight replicate plates were prepared using standard template format and HT-29 cells. For EA 1 hour pre-incubation, three sets of duplicate plates were prepared, each set receiving a given dose of the inhibitor (90, 30, and 10 μM). Each plate was incubated in the presence of Dox for 4 hours. At the end of this period, culture medium was removed and replaced with fresh drug-free medium. Data points represent the mean ± SD of sextuplicate determinations from the duplicate plates (n=12). Dashed line to the y-axis represents toxicity in the absence of Dox.

6.1.2.5.2 TER 199, 183, 206

For combination studies employing the Terrapin GST inhibitors, a 4 hour pre-incubation was used in place of the 1 hour period used for EA. This longer pre-incubation was necessary as the parent Terrapin compounds are not in their active state (as was the case for EA). TER 183, 199 and 206 are all diethyl esters of the active compound. This chemical modification allows the agents to enter the cell passively, but requires de-esterification once inside the cell for the agent to become

active (Ciaccio et al., 1995; Morgan et al., 1996). Following the pre-incubation, Dox was added to the culture wells in a similar fashion as was performed for EA.

The doses of GST inhibitor used for the MTT assay were selected based on a combination of the results of the dose-response curves for toxicity of the inhibitors alone and the reported *in vitro* K_is of the free acid versions of the inhibitors: TER 199 K_i for GST P1 = 0.45 μM, TER 183 K_i for GST A1 = 0.27 μM (published and unpublished data, Terrapin Inc.). Combination studies were performed with serial dilutions of Dox on duplicate 96-well plates. Each set of duplicates was exposed to a single dose of inhibitor. Generally, 4 sets of 2 plates were prepared and various concentrations of inhibitor were applied. A single set of 2 plates was used as control (Dox alone).

As with the EA study, the time frame of drug exposure was assessed for its impact on the ability of the GST inhibitors to cause Dox potentiation. Initially a 4 hour pre-incubation of inhibitor followed by a 4 hour co-incubation with Dox was employed. At the end of this period Dox was removed and inhibitor was replaced at the original concentration. Figures 12 and 13 show typical responses of the cells to the combination treatments.

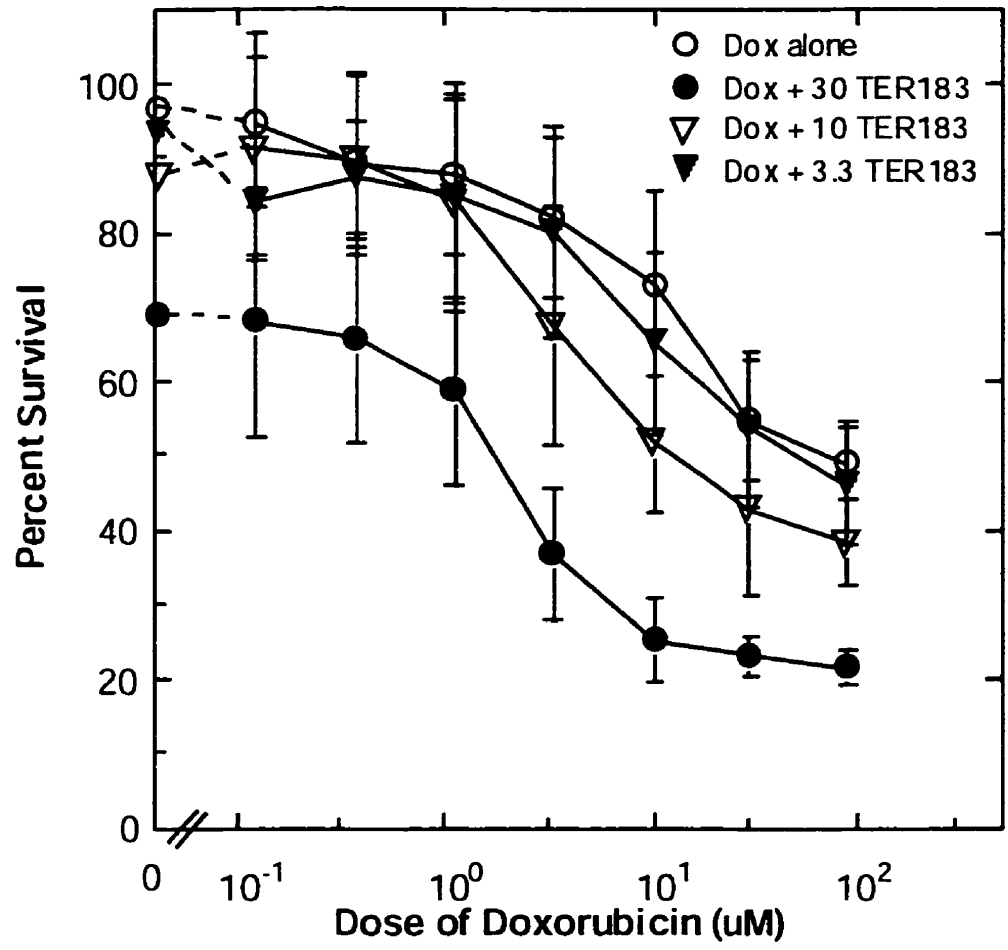

Figure 12: Effect of GST Alpha inhibitor TER 183 on Dox toxicity in Caco-2 cells

Eight replicate plates were prepared using standard template format and Caco-2 cells. Each plate received identical Dox exposure for 4 hours. For TER 183 four hour pre-incubation, three sets of duplicate plates were prepared, each set receiving a given dose of the inhibitor at 30, 10 and 3.3 μM. At the end of the Dox incubation, culture medium was removed and replaced with fresh medium containing TER 183 at the original pre-incubation concentration. Cells were maintained in this medium for the duration of the assay. Data points represent the mean ± SD of sextuplicate determinations from duplicate plates (n=12). Dashed line to the y-axis represents toxicity in the absence of Dox

Each of the four cell lines was systematically evaluated for the ability of either TER 183 or 199 to effect potentiation of the cytotoxic effects of Dox, by inhibiting GST Alpha or Pi respectively. Doses were optimized to attempt to maximize GST inhibition while limiting the toxicity resulting from the test compound alone. While a number of isolated experiments did show evidence of toxicity that was greater than the sum of the individual effects of the inhibitor and the antineoplastic, the most consistent effect

was by far additive toxicity. In addition to the standard experimental protocol, assays with a modified time-course (i.e. chronic inhibitor - chronic Dox; acute inhibitor - acute Dox) were also attempted before concluding that GST inhibition was not resulting in potentiation of colon cancer cell cytotoxicity when used in conjunction with Dox.

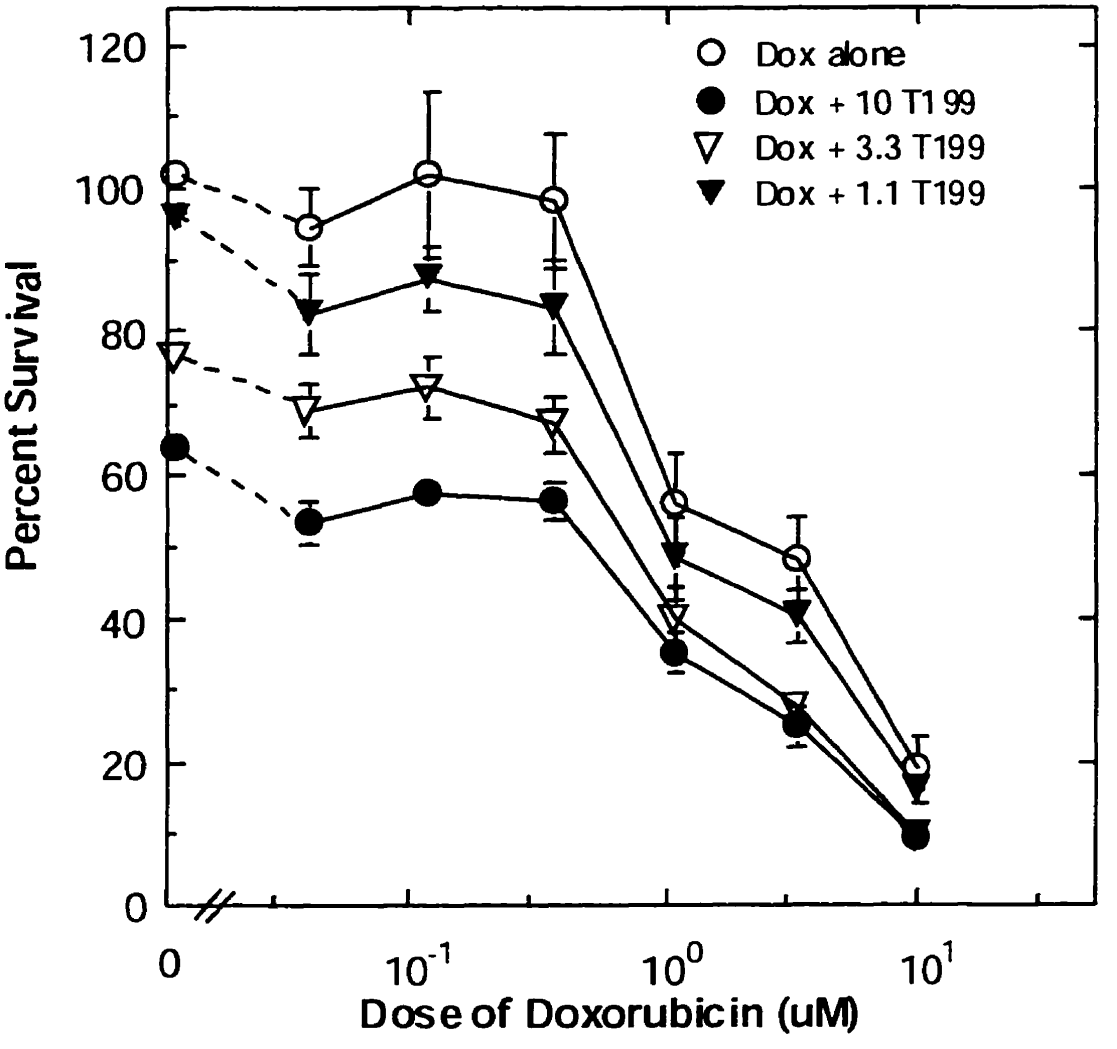

Figure 13: Effect of GST Pi inhibitor TER 199 on Dox toxicity in HT-29 cells

Eight replicate plates were prepared using standard template format and HT-29 cells. Each plate received identical Dox exposure for 4 hours. For TER 199 four hour pre-incubation, three sets of duplicate plates were prepared, each set receiving a given dose of the inhibitor at 10, 3.3, and 1.1 μM. At the end of the Dox incubation, culture medium was removed and replaced with fresh medium containing TER 199 at the original pre-incubation concentration. Cells were maintained in this medium for the duration of the assay. Data points represent the mean ± SD of sextuplicate determinations from duplicate plates (n=12). Dashed line to the y-axis represents toxicity in the absence of Dox.

As neither EA nor the selective inhibitors of GSTs Alpha and Pi potentiated the effects of the anthracycline Dox, we turned to the assessment of combinations of the GST inhibitors with an alkylating agent. Previous studies had indicated that the toxicity of the alkylating agent chlorambucil, (which is known to be primarily conjugated to GSH by GST Alpha and to some extent by GST Pi) could be potentiated by inhibitors of GST, such as EA (Tew et al., 1988; Ciaccio et al., 1991; Morgan et al., 1996). It has been observed that TER 199 is able to potentiate, by greater than 2-fold the toxicity of Chlor to an EA-sensitive derivative of HT-29 (HT4-1 (Kuzmich et al., 1992)), and also that TER 183 is able to produce lesser but still significant potentiation to the same line (Morgan et al., 1996). Chlor toxicity to the parental HT-29 cell line has been reported to be potentiated by about 2-fold by TER 199. We utilized very similar combinations of Chlor in conjunction with both Terrapin compounds and EA, but were unable to match the degree of potentiation previously reported (Morgan et al., 1996). Figure 14, which is the result of only a single assay, shows a very slight increase (if any) in toxicity for the combination treatment with EA, compared to treatment with alkylating agent alone. Similar assays with combinations of TER 199 (Figure 15) and TER 183 (data not shown) failed to show any significant potentiation.

TER 206 was not assayed for potentiation of Dox toxicity based on the lack of GST Mu protein or conjugative activity in these colon cell lines.

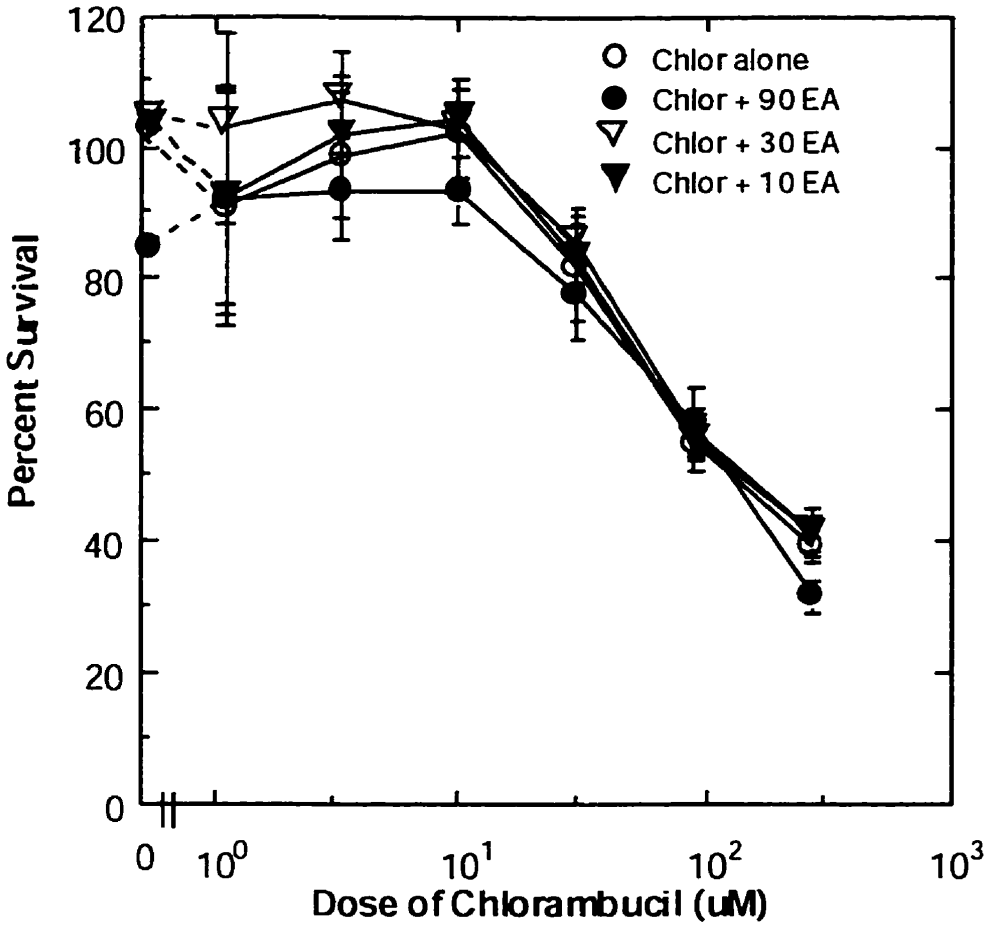

Figure 14: Effect of EA on chlorambucil toxicity in HT-29 cells

Eight replicate plates were prepared using standard template format and HT-29 cells. Each plate received identical Dox exposure for 4 hours. For EA 4 hour pre-incubation, three sets of duplicate plates were prepared, each set receiving a given dose of the inhibitor at 90,30, and 10 μM. At the end of the Chlor incubation, culture medium was removed and replaced with fresh medium containing EA at the original pre-incubation concentration. Cells were maintained in this medium for the duration of the assay. Data points represent the mean ± SD of sextuplicate determinations from duplicate plates (n=12). Dashed line to the y-axis represents toxicity in the absence of Chlor.

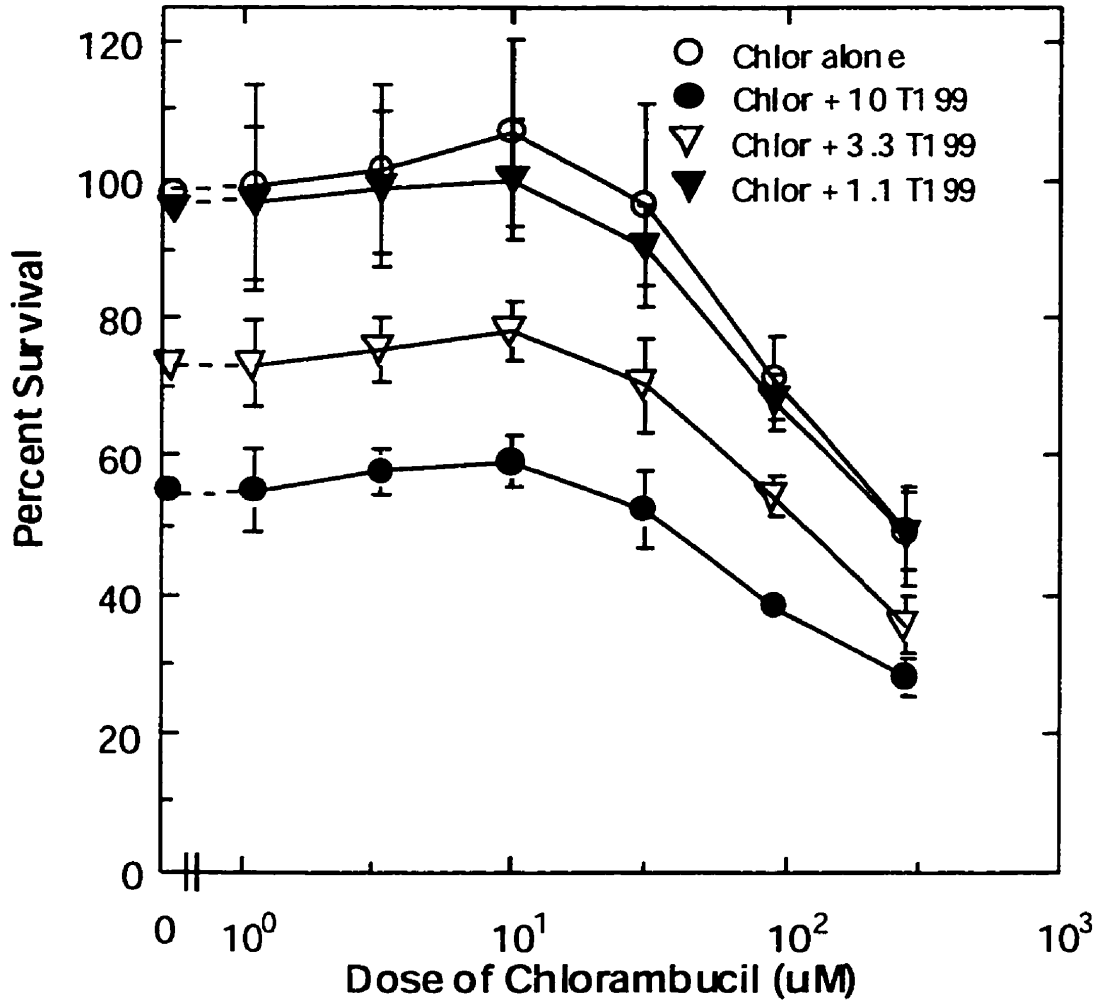

Figure 15: Effect of GST Pi inhibitor TER 199 on chlorambucil toxicity in HT-29 cells.

Eight replicate plates were prepared using standard template format and HT-29 cells. Each plate received identical chlorambucil exposure for 4 hours. For TER 199 4 hour pre-incubation, three sets of duplicate plates were prepared, each set receiving a given dose of the inhibitor at 10, 3.3, and 1.1 μM. At the end of the chlorambucil incubation, culture medium was removed and replaced with fresh medium containing TER 199 at the original pre-incubation concentration. Cells were maintained in this medium for the duration of the assay. Data points represent the mean ± SD of sextuplicate determinations from duplicate plates (n=12). Dashed line to the y-axis represents toxicity in the absence of Chlor.

6.1.3 Colony Formation Assay

Subsequent to the failure of our microculture colourimetric assay to show any significant potentiation with either anthracycline or alkylating agent - GST inhibitor combinations, we turned to the colony formation assay to verify these results. This assay, while comparable to the MTT for determination of response to chemotherapy, is generally perceived as being more sensitive and can detect cytotoxicity over a much

larger range (3 to 4 logs of cell kill). A limited number of these assays were performed on a single colon cancer cell line (HT-29) using antineoplastics alone and in combination with GST inhibitors.

6.1.3.1 Antineoplastic Toxicity

Simple dose response curves for inhibition of colony formation for both Dox and Chlor were produced for HT-29 cells. As can be seen in Figure 16, the results for HT-29 cytotoxicity due to Dox are comparable between MTT and clonogenic assays. The slope of the dose-response curve shows reasonable similarity to that seen in Figure 7. Initiation of toxicity, IC_{50} and IC_{90} are all approximately 0.8 - 1.0 log units lower in this assay.

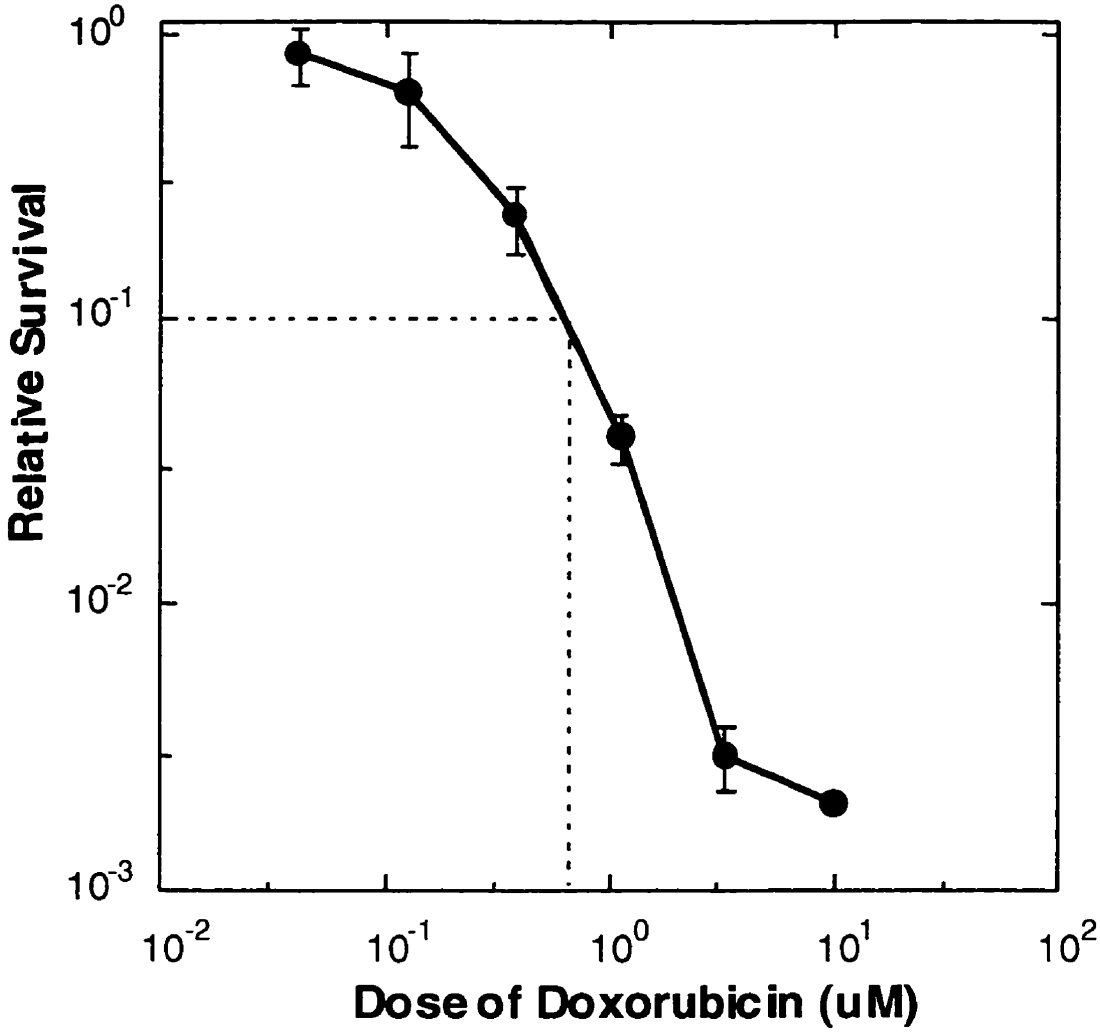

Figure 16: Dox cytotoxicty in HT-29 cells as measured by colony forming assay

Data points represent mean relative survival ± SD of triplicate to sextuplicate determinations in a single colony formation assay. Relative survival of 10^0 is the mean number of colonies observed in growth wells cultured in the absence of Dox. Dashed line indicates the dose of Dox that results in 90% inhibition of colony formation (IC_{90} = 0.62 uM).

Chlor, like Dox, also has some of its common descriptive toxicity points (IC_{50}, IC_{75}, etc.) occurring at about one log unit lower dose in this assay (Figure 17), than in the MTT (Figure 8). The absolute concentration of Chlor at the point where toxicity is initially evident, is about 2 orders of magnitude greater than for Dox. This is also observed under the MTT assay. The response of the HT-29 cell line up to the point of about 50% toxicity is similar between the two drugs and the two assays (i.e. slope of

the dose response curve). Below this point, however, it is interesting to note that although Chlor may initiate toxicity at 100-fold greater concentration than Dox, the clonogenic assay reveals that this difference becomes less pronounced with increasing dose. At IC_{90} this difference has been reduced to 50-fold and even further to 25-fold at IC_{99}. Determination of these values by MTT was not possible due to the lower limit of detection of the MTT assay and an inability to achieve the necessary concentrations in solution.

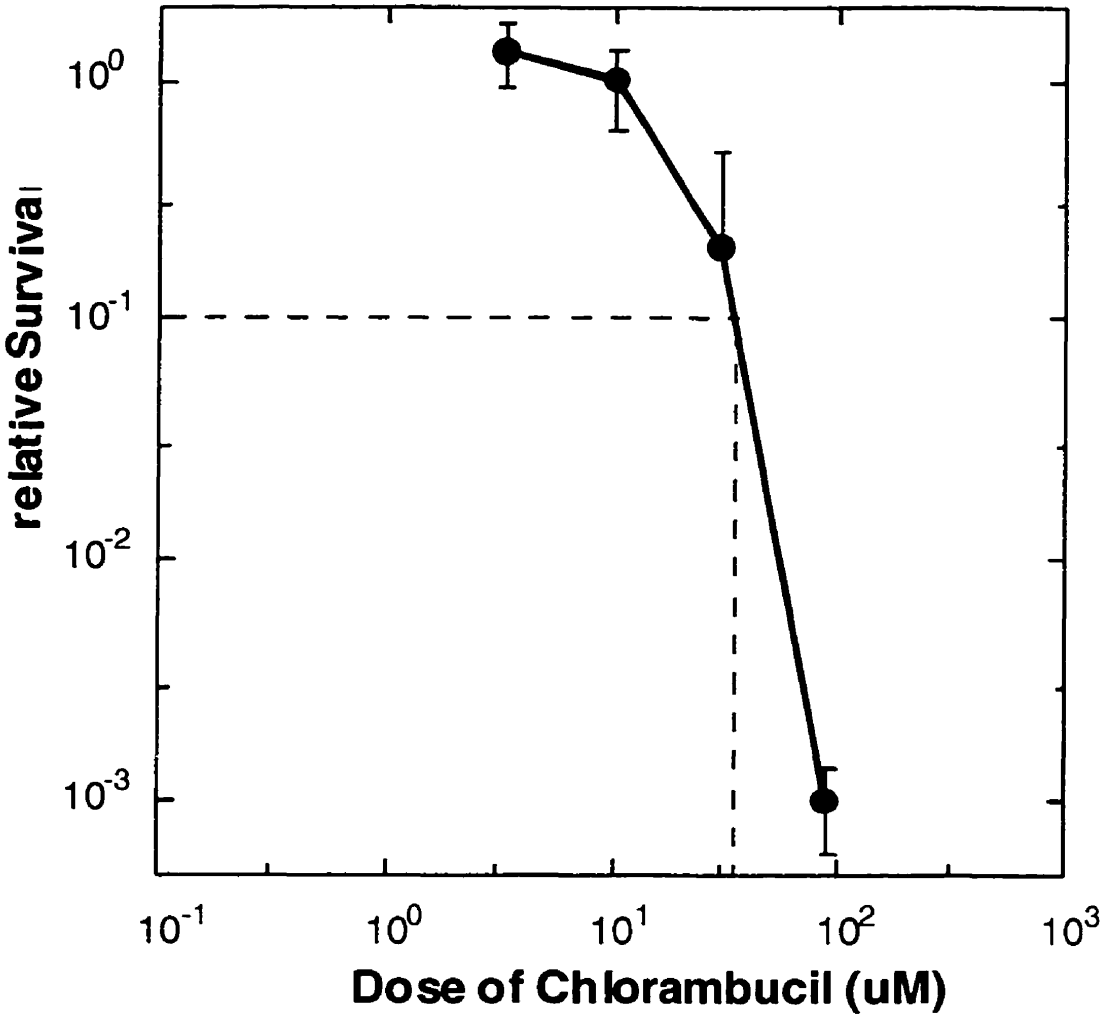

Figure 17: Chlorambucil cytotoxicty in HT-29 cells as measured by colony forming assay

Data points represent mean relative survival ± SD of triplicate to sextuplicate determinations in a single colony formation assay. Relative survival of 10^0 is the mean number of colonies observed in growth wells cultured in the absence of Chlor. Dashed line indicates the dose of Chlor that results in 90% inhibition of colony formation (IC_{90} = 33 uM).

6.1.3.2 Combination Studies

Dox or Chlor were combined with the GST Pi selective inhibitor TER 199 in separate colony formation assays to investigate if potentiation is occurring but has not yet been detected due to the limitations of our colourimetric assay. Figure 18 shows a combination study employing Dox and the GST Pi selective inhibitor TER 199. Under the MTT assay there was no evidence of potentiation seen in any of the cell lines

despite significant toxicity due to effects of the inhibitor alone. While there is still no evidence of potentiation in this cell line for Dox, there is a curious lack of toxicity due to the Terrapin inhibitor. Preliminary clonogenic assays conducted solely with TER 199 at multiple doses, while not conducted satisfactorily at 30 μM, did show a trend for much reduced toxicity due to the inhibitor in the clonogenic vs. MTT assay (data not shown).

Assessment of the effect of TER 199 on Chlor toxicity produced the only suggestive evidence of potentiation in our study. Again using the HT-29 cell line, in which Chlor toxicity has been reported to be potentiated by about 2-fold by this GST Pi inhibitor (Morgan et al., 1996), we observed an increase in the toxicity of Chlor at doses of inhibitor that were completely non-toxic to the cell line. At doses of Chlor which decreased colony formation to 1.5% of control, the addition of either 3 or 30 μM of TER 199 reduced cloning ability to 0.3%, an increase in toxicity of nearly 5-fold. Effects at this low degree of cell survival were not measurable using our colourimetric assay. It must be pointed out that this evidence for potentiation of Chlor toxicity by TER 199 is derived from a single point on a dose-survival curve.

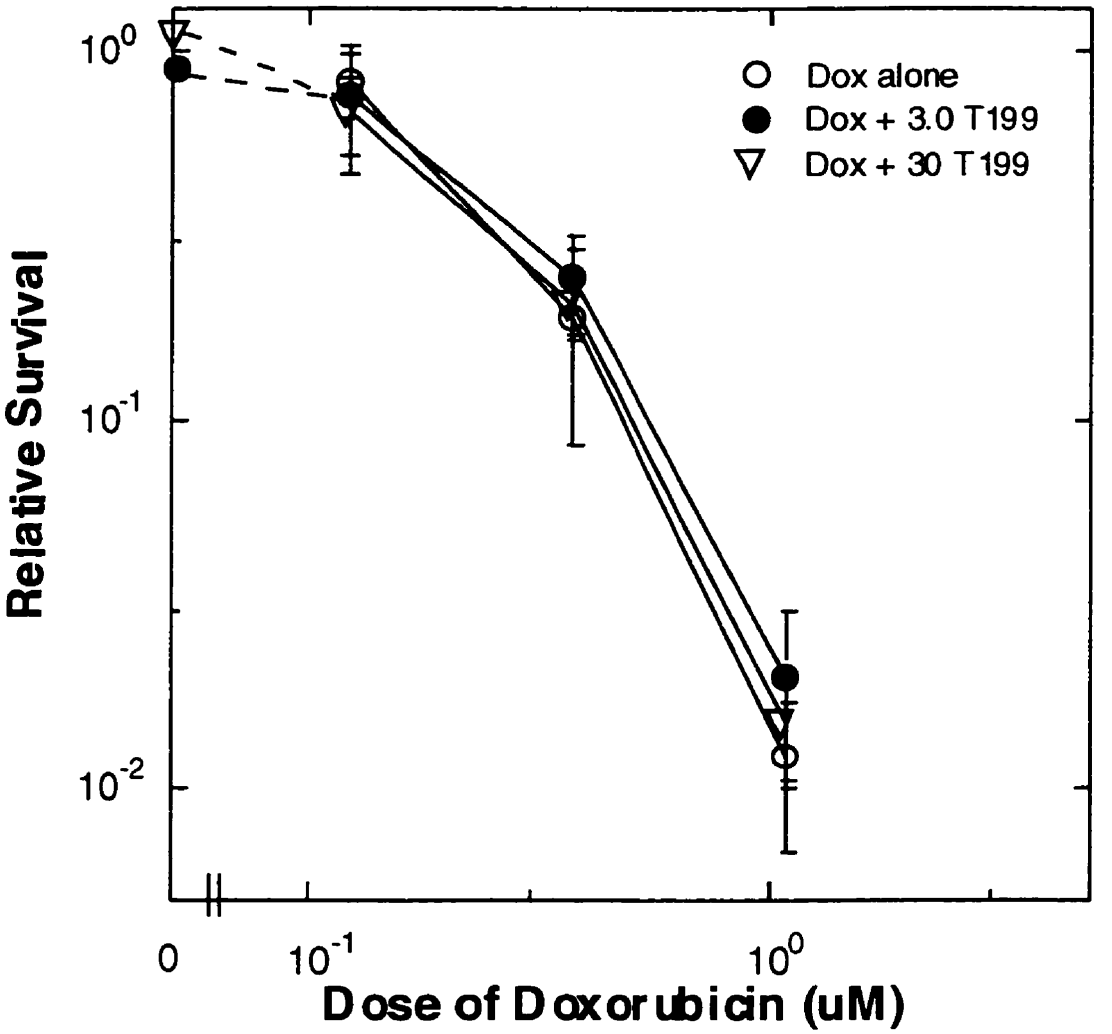

Figure 18: Effect of GST Pi inhibitor TER 199 on Dox cytotoxicity as measured by colony forming assay

Results of a combination study with 4 hour pre-incubation of TER 199 (T199) and a further 4 hour incubation in conjunction with Dox using the HT-29 cell line. Data points represent mean ± SD of triplicate to sextuplicate determinations in a single colony formation assay. Relative survival of 10^0 is the mean number of colonies observed in growth wells cultured in the absence of Dox. Dashed lines to the y-axis represents toxicity in the absence of Dox.

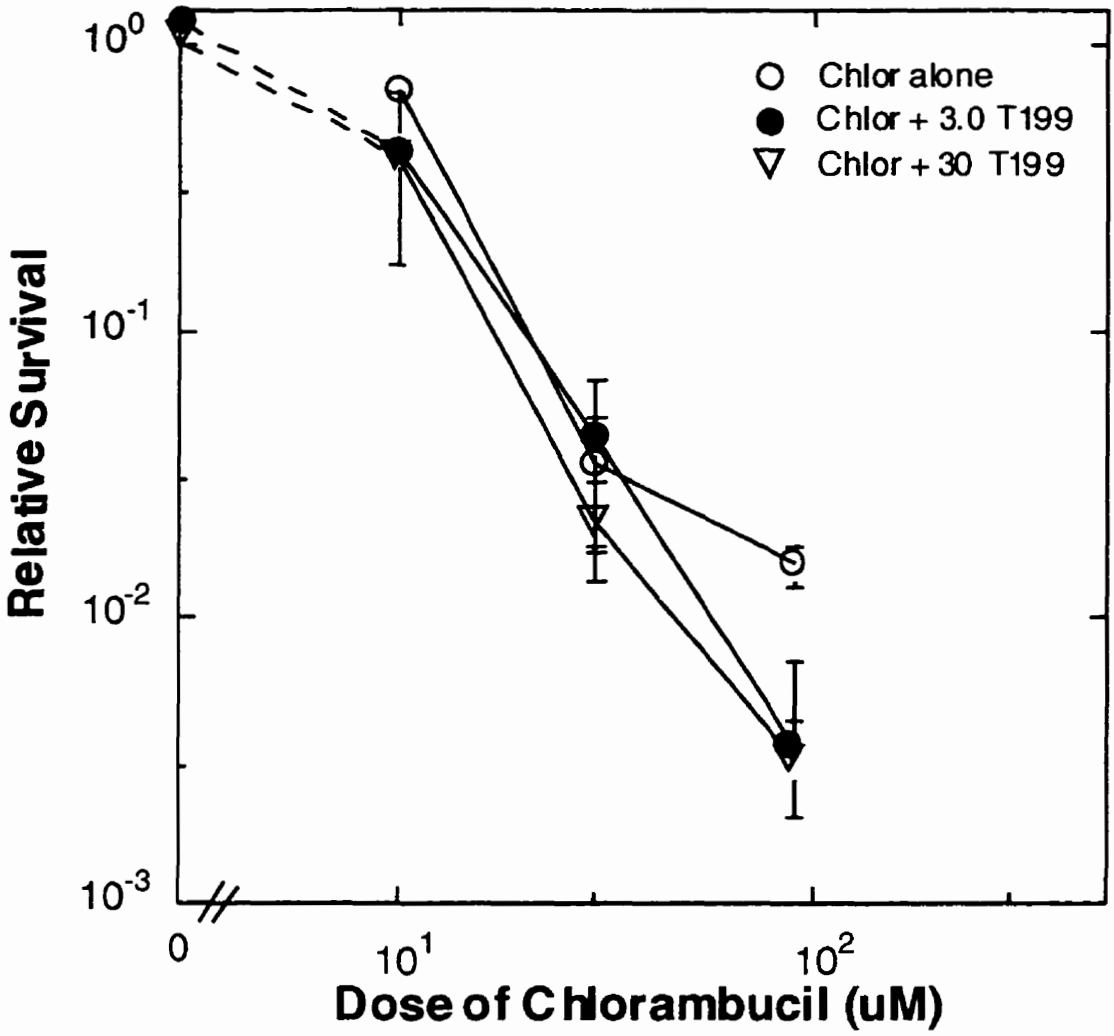

Figure 19: Effect of GST Pi inhibitor TER 199 on chlorambucil cytotoxicity as measured by colony forming assay

Results of a combination study with 4 hour pre-incubation of TER 199 (T199) and a further 4 hour incubation in conjunction with Chlor using the HT-29 cell line. Data points represent mean relative survival ± SD of triplicate to sextuplicate determinations in a single colony formation assay. Relative survival of 10^0 is the mean number of colonies observed in growth wells cultured in the absence of Chlor. Dashed lines to the y-axis represents toxicity in the absence of Chlor

6.2 Western Blotting

6.2.1 GST Pi

To assay for the presence and relative quantity of GST Pi immunoreactive protein in cytosolic samples, a western blot analysis was performed. As can be seen in Figure 20, the GST Pi polyclonal antibody recognizes a single band of approximate molecular weight of 25 kDa in all cell lines. Each colon cell line shows comparable

immunoreactivity both between cytosol samples and cell lines. Laser densitometry, under conditions that gave a linear relationship between cytosolic protein and densitometric response, showed no significant difference in GST Pi protein between these cell lines.

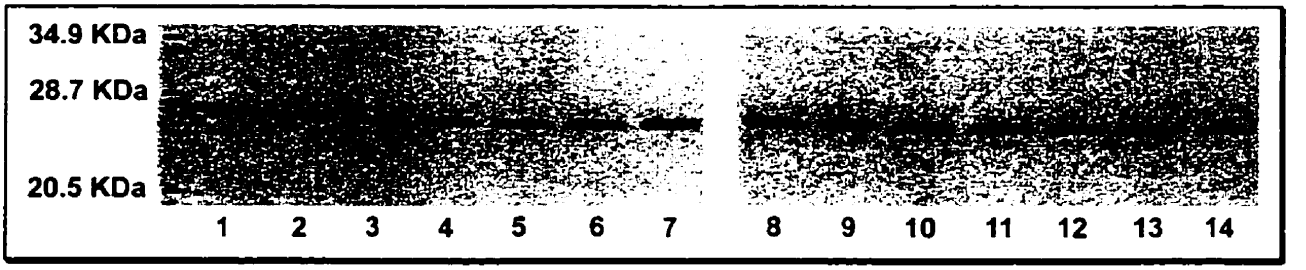

Figure 20: GST Pi protein expression in colon cancer cell lines

Immunoblot produced by 6 μg of protein, electrophoresed on a 12% acrylamide gel. Cytosol samples were diluted to approximately 1 mg/ml and verified by Bradford protein assay. Volumes were calculated to load exactly 6 μg of protein per lane in triplicate or quadruplicate samples. The Hybond ECL membrane (Amersham) was incubated with 1:50,000 primary anti-GST Pi antibody (Biotrin) and 1:5,000 secondary HRP conjugate(Amersham). Using enhanced chemiluminescence the membrane was exposed to Kodak film for 6 minutes and then developed. 3 or 4 independent samples of each cytosol were electrophoresed on two gels. In the autoradiograph above: Lanes 1-4: SW620 (10.13±1.45), Lanes 5-7: HT-29 (12.51±3.52), Lanes 8-11: Caco-2 (13.58±3.33), Lanes 12-14: LoVo (8.13±1.25). Values in parentheses represent mean± SD of the densitometric values in arbitrary units. One-way ANOVA P=0.08.

6.2.2 GST Alpha

A western blot was performed on samples of colon cell cytosol to assay for GST Alpha immunoreactive protein. Unlike GST Pi, the Alpha class is composed of at least two isozymes and possibly as many as six [as reviewed in (Hayes and Pulford, 1996)], hence, we employed the full-size gel to increase the resolution of the multiple bands which may be present and, which likely migrate at only minimally different molecular weight (MW). In addition to pre-stained MW markers (Bio-Rad) which are of use in visualizing the progress of electrophoretic separation, we utilized unstained markers on these gels which have in our experience proven to be more accurate indicators of MW. Also, in addition to the triplicate cytosol samples from the four cell

lines, purified GST A1, A2 and P1 proteins were electrophoresed in the gel to validate that the band(s) migrated to the appropriate MW for GST Alpha. Using a polyclonal antibody directed against human GST Alpha (Biotrin) the Caco-2 cell cytosol produced a single immunoreactive band of similar intensity (visual observation only) to 0.01 µg of purified GST A1 or GST A2. The band migrated to approximately the same position as the purified proteins and was not present in any of the other cytosol samples. A second band, however, of slightly lower MW, was present in all colon cytosols. This band was heterogeneous in intensity, both between colon cell lines and among the triplicate samples. In all cases it was significantly less intense than the main band seen in Caco-2. This band was not recognized in any of the GST Alpha or Pi purified protein lanes. These results were confirmed on a replicate immunoblot incubated with an independent antibody to GST Alpha (Dr. Ken Tew). The film produced from this blot confirms that Caco-2 cells highly express an immunoreactive protein that co-migrates with GST A1 and A2, and that the protein is unique among the colon cancer cell lines assayed. The second, lower MW band was not recognized by this new antibody (data not shown). Densitometry was not performed on either of the GST Alpha immunoblots due to the obvious differences between Caco-2 and the remaining three cell lines.

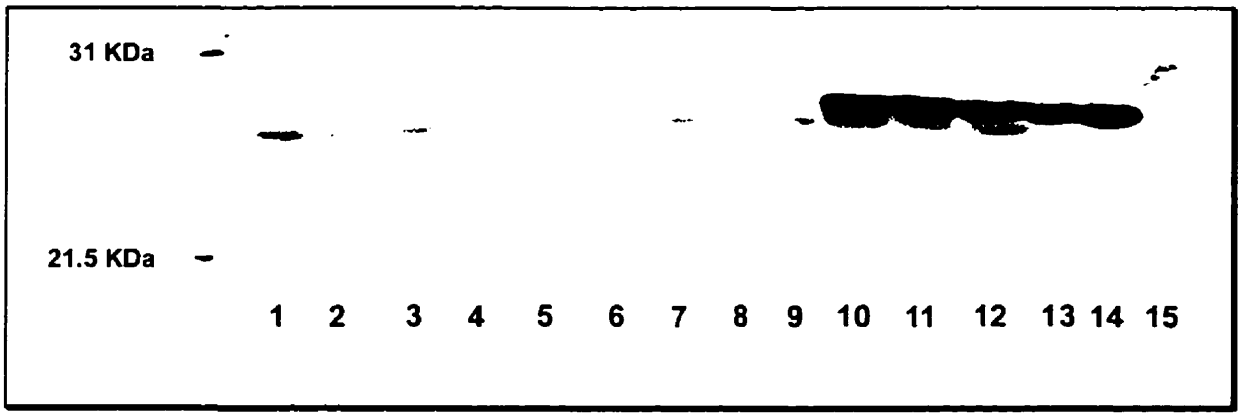

Figure 21: GST Alpha protein expression in colon cancer cell lines

Immunoblot produced by 20 µg of protein, electrophoresed on a full size 12% acrylamide gel. Cytosol samples were diluted to approximately 1 mg/ml and verified by Bradford protein assay. Volumes were calculated to load exactly 20 µg of protein per lane in triplicate samples. The Hybond ECL membrane (Amersham) was incubated with 1:1000 primary anti-GST Alpha antibody (Biotrin) and 1:1,000 secondary HRP conjugate(Amersham). Using enhanced chemiluminescence the membrane was exposed to Kodak film for 1 minute and then developed. Three independent samples of each cytosol were electrophoresed for each cell line. In the autoradiograph above: Lanes 1-3: HT-29, Lanes 4-6: SW620, Lanes 7-9 LoVo, Lanes 10-12 Caco-2, Lane 13 GST A1, Lane 14 GST A2, Lane 15 GST P1 (0.01 µg of purified protein)

6.2.3 GST Mu

No specific GST Mu immunoreactive band was detected in any of the four cell lines (data not shown).

6.2.4 MRP

If the conjugation of Dox, Dox metabolites or products of lipid peroxidation by GST, or if the non-selenium dependent GST-based reduction of GSH to GSSG is an integral component of intrinsic drug resistance observed in colon cancer, it would seem reasonable that there would be present a mechanism to extrude these conjugates from the cell for ultimate excretion from the body. To this end, a western blot was performed to determine if MRP (the putative GSH-conjugate pump) was present in colon cancer cells. Figure 22 is an image of the film produced from this western blot. Although there is a single band visible on the film at approximately 95

kDa no immunoreactive signal is present in the 190 kDa region (the expected size of MRP). This 95 kDa band has been seen by other investigators using the MRPm6 antibody under similar assay conditions. Its presence has been attributed to cross-reactivity of the donkey-anti-mouse immunoglobulin secondary antibody with other proteins present in the membrane fraction of human cell lines (Flens et al., 1996). Hence, these results show no evidence for expression of the MRP protein in these cell lines.

Figure 22: MRP protein expression in colon cancer cell lines

A 7.5 % acrylamide SDS-PAGE mini-gel was prepared and electrophoresed with 24 µg of protein in triplicate samples of the membrane pellet from differential centrifugation preparations of each cell line, along with stained and unstained molecular weight markers. Primary anti-MRPm6 antibody was employed at 1:20 dilution for 2 hours at room temperature, followed by 1:1000 anti-mouse HRP conjugate for 1 hr at room temperature, with appropriate washing steps. The membrane was developed with ECL detection reagents and exposed to Kodak film overnight. In the autoradiograph above: Lanes 1-3 HT-29, Lanes 4-6 Caco-2, Lanes 7-9 SW620, Lanes 10-12 LoVo.

6.3 Northern Blotting

Analysis of GST mRNA was performed on all cell lines in order to assess for variability in transcript levels and also for evaluation of which specific GST transcripts are present. This isozyme-specific analysis was not possible by western blot with our polyclonal antibodies. Therefore, several DNA oligonucleotide probes were synthesized (for sequences see Methods section 5.7.2) which were specific for human GST P1, A1, A2, M1, M2 and M3. A single northern blot was prepared which

contained RNA from each of the cell lines. This one blot was hybridized sequentially with each of the radiolabeled oligonucleotides, exposed to autoradiographic film, and then stripped and re-hybridized with the next probe. In addition to the GST oligonucleotides, the blot was probed with an oligonucleotide for the housekeeping gene α-tubulin. This allowed normalization of the quantities of GST mRNA present in these individual cell lines.

6.3.1 GST P1

The autoradiograph shown in Figure 23 indicates that GST P1 mRNA transcript (0.75 kb) is present in each of the colon cell lines and that it is detectable with as little as 5 μg of total RNA. Quantitation was performed by laser densitometry and the values were divided by the corresponding readings produced from densitometric analysis of the tubulin mRNA blot. This data analysis produced the following levels of GST Pi / α-tubulin expression (in arbitrary units from lowest to highest): SW620 - 0.29; LoVo - 0.39; HT-29 - 0.42; and Caco-2 - 0.54.

6.3.2 GST A1 and A2

Figure 23 also shows the faint signal produced by the GST A2 oligonucleotide. This band in the ~0.9 kb region is visible only in the Caco-2 lane loaded with 15 μg of RNA. The band was not visible for 5 or 10 μg of RNA and was not present in any other cell line. Northern hybridization with GST A1 oligonucleotide probe showed no detectable signal in the relevant 0.75 kb region even after 5 days of exposure and production of a moderate amount of background signal (data not shown). Quantitation of GST A2 mRNA was not performed as it was only visible in a single cell line.

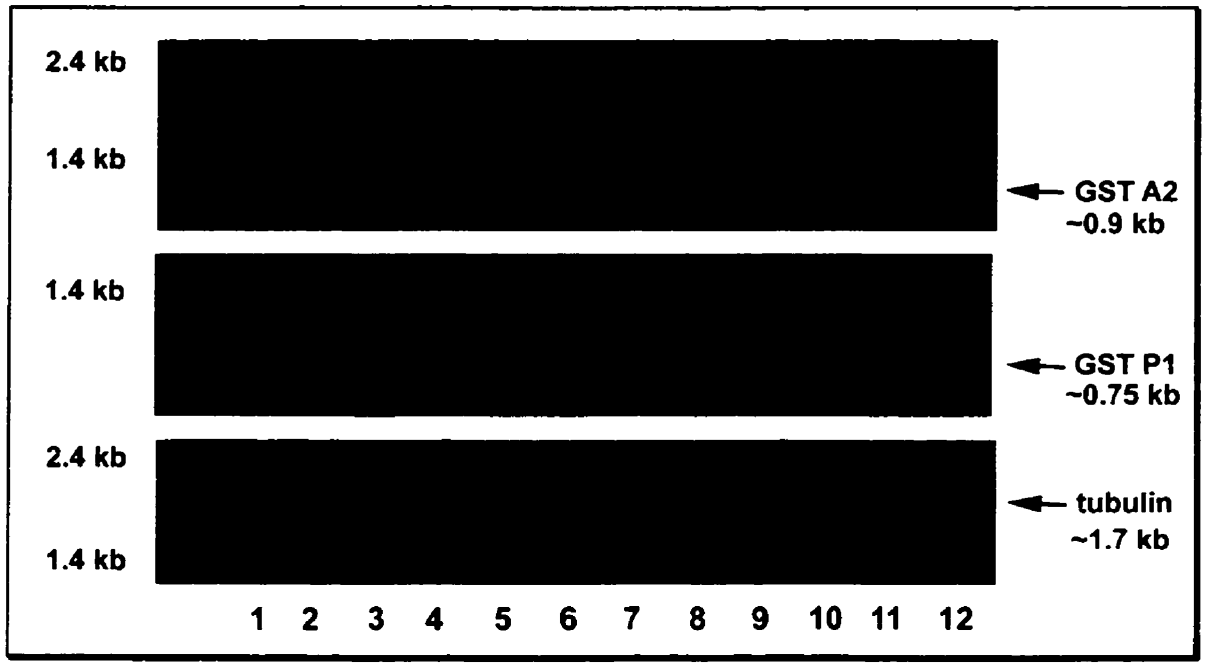

Figure 23: GST mRNA expression in colon cancer cell lines

Autoradiograph of a northern blot sequentially probed with ^{32}P-labeled oligonucleotides specific for GST A2, GST P1& α-tubulin sequences. Exposure was for 5 days each. 5, 10 and 15 µg of RNA from each of four cell lines are in lanes: 1-3, Caco-2; 4-6, LoVo; 7-9, SW620; 10-12, HT-29.

6.3.3 GST M1, M2, M3

Northern blot hybridization with GST M1, M2 and M3 oligonucleotide probes did not detect these transcripts in any of the four cell lines (data not shown).

6.4 GST Catalytic Activity

6.4.1 Total GST activity

The assay for total GST activity using CDNB as substrate was optimized with respect to CDNB and GSH concentrations, as was the linearity of the reaction, with respect to protein and time, by other members of our laboratory, using LS180 cytosol. For comparison of total conjugative activity between Caco-2, SW620, LoVo, and HT-29 cytosols, three to four separate cytosolic samples from each line were assayed in triplicate. The bar graph in Figure 24 illustrates the CDNB conjugation activity for all cell lines. Caco-2 cytosol was found to have the highest activity at 412 nmol/min/mg

protein compared to HT-29, SW620 and LoVo which had activities of 229, 245 and 277 nmol/min/mg of protein, respectively. The CDNB conjugation activity of Caco-2 cytosol was significantly higher than all other cell lines.

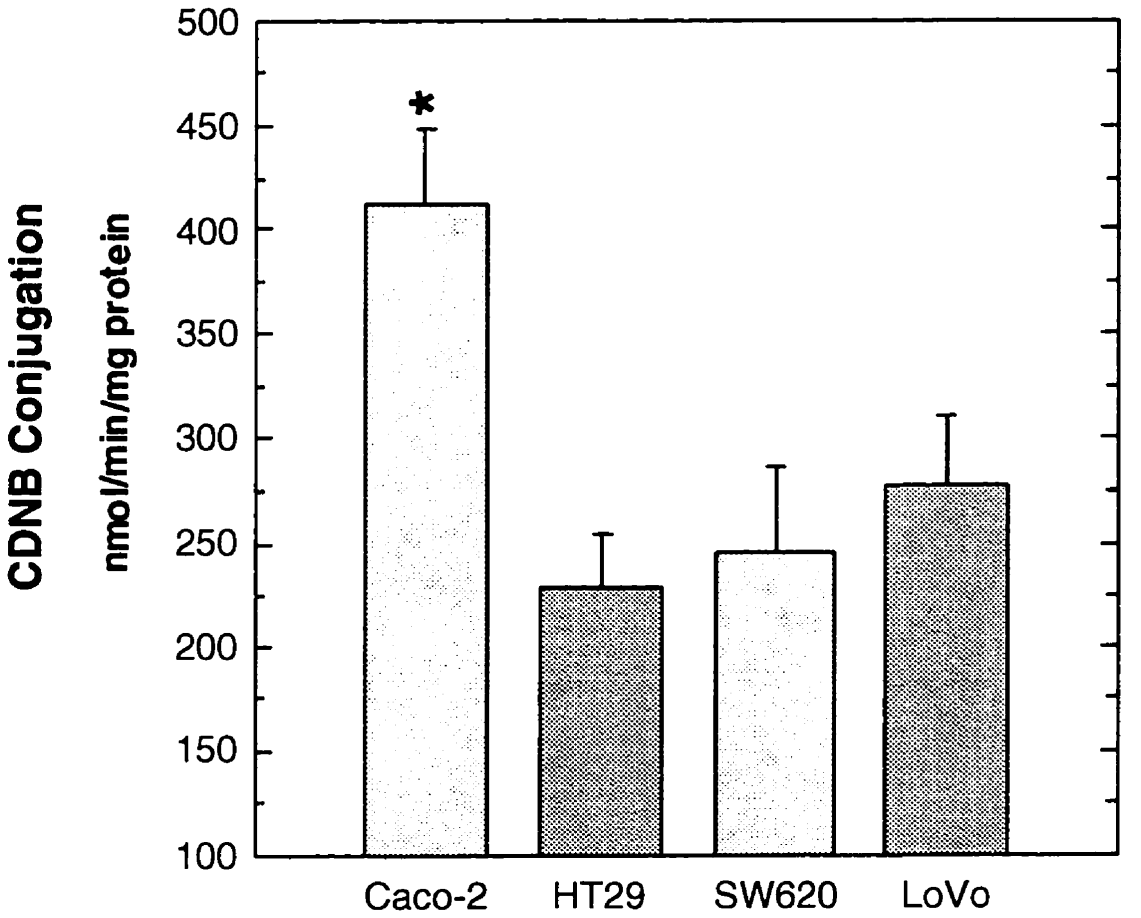

Figure 24: CDNB conjugation as a marker for total GST activity

Conjugation of GSH using CDNB as substrate, measured spectrophotometrically. Bars represent mean ± SD of N=3 or 4 independent samples assayed in triplicate for each cell line. Asterisk (*): One way ANOVA detected a significant ($P < 0.0001$) variation in activities among the cell lines. Post-hoc Student-Newman-Keuls tests revealed Caco-2 had significantly ($P<0.001$) greater activity than all other cell lines.

6.4.2 GST Pi Selective Activity

As GST Pi is implicated as a factor in antineoplastic drug resistance (Mekhail-Ishak et al., 1989; Peters et al., 1989; Clapper et al., 1991), the contribution made by this enzyme to the total GST conjugative activity was assessed using ethacrynic acid (EA) as a GST Pi class selective substrate. Experiments were conducted on three

individual cytosol samples for each cell line, and the assay was performed in triplicate. A bar graph illustrating the GST Pi selective activity is shown in Figure 25, which clearly shows that there is no significant difference in GST Pi selective catalytic activity among the cell lines.

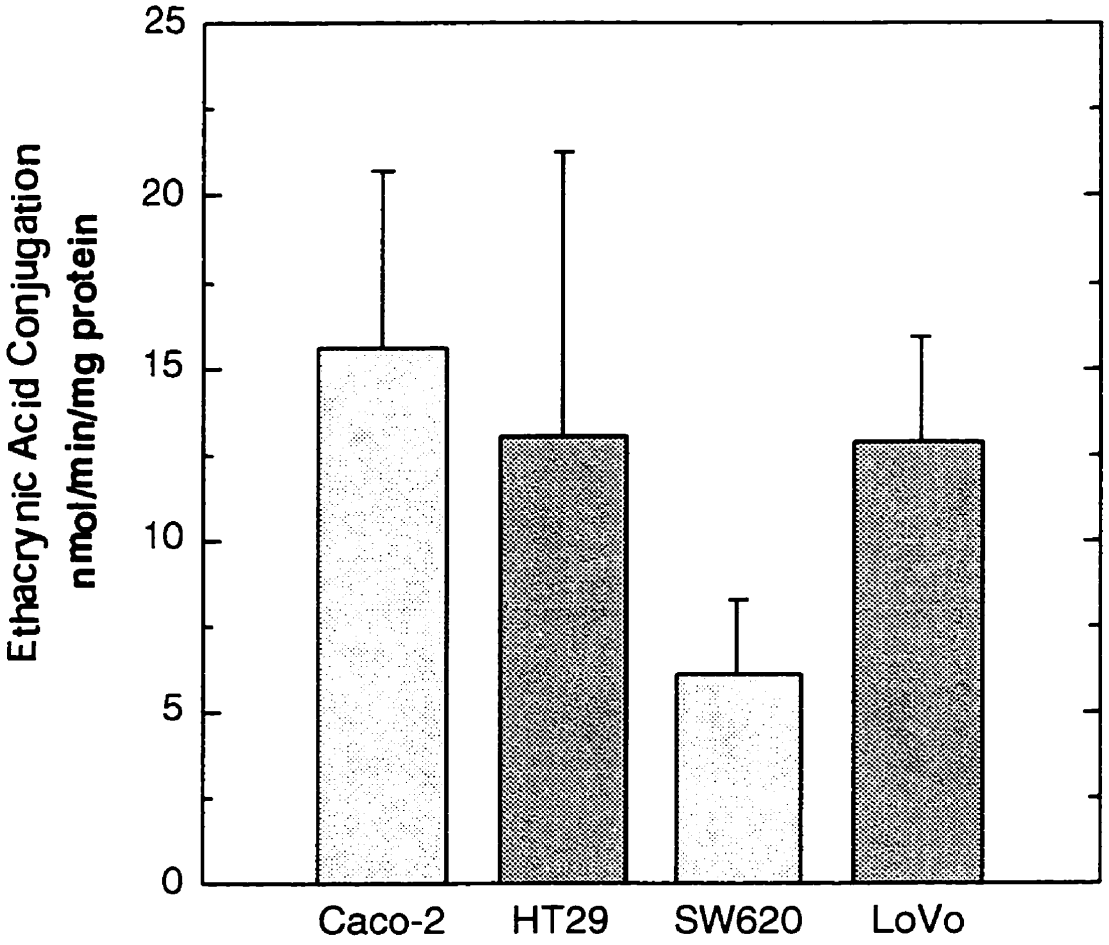

Figure 25: Ethacrynic acid conjugation as a marker for GST Pi activity

EA is used as a substrate to measure GST Pi. Bars represent the mean ± SD of three samples assayed in triplicate for each cell line. No significant difference among cell lines was detected by one-way ANOVA (P=0.22)

6.4.3 GST Alpha Selective Activity

To selectively measure the GST Alpha activity in these cell lines, a unique feature of the Alpha class enzymes was exploited. Among the GSTs expressed in colon cancer, GST Alpha forms possess the highest activity as selenium-independent GSH peroxidases. Specifically, the activity is directed toward organic hydroperoxides,

such as cumene hydroperoxide (CHP). To this end, the method of Lawrence and Burk (1976) was employed to selectively measure GST Alpha activity. The results of this assay are displayed in Figure 26 and were very much unexpected, and are not in agreement with our assessment of GST Alpha protein and mRNA in these cell lines. The observation that HT-29 and SW620 cells possess the greatest amount of GST Alpha activity and Caco-2 the least, appears to contradict our assessment of GST protein and mRNA.

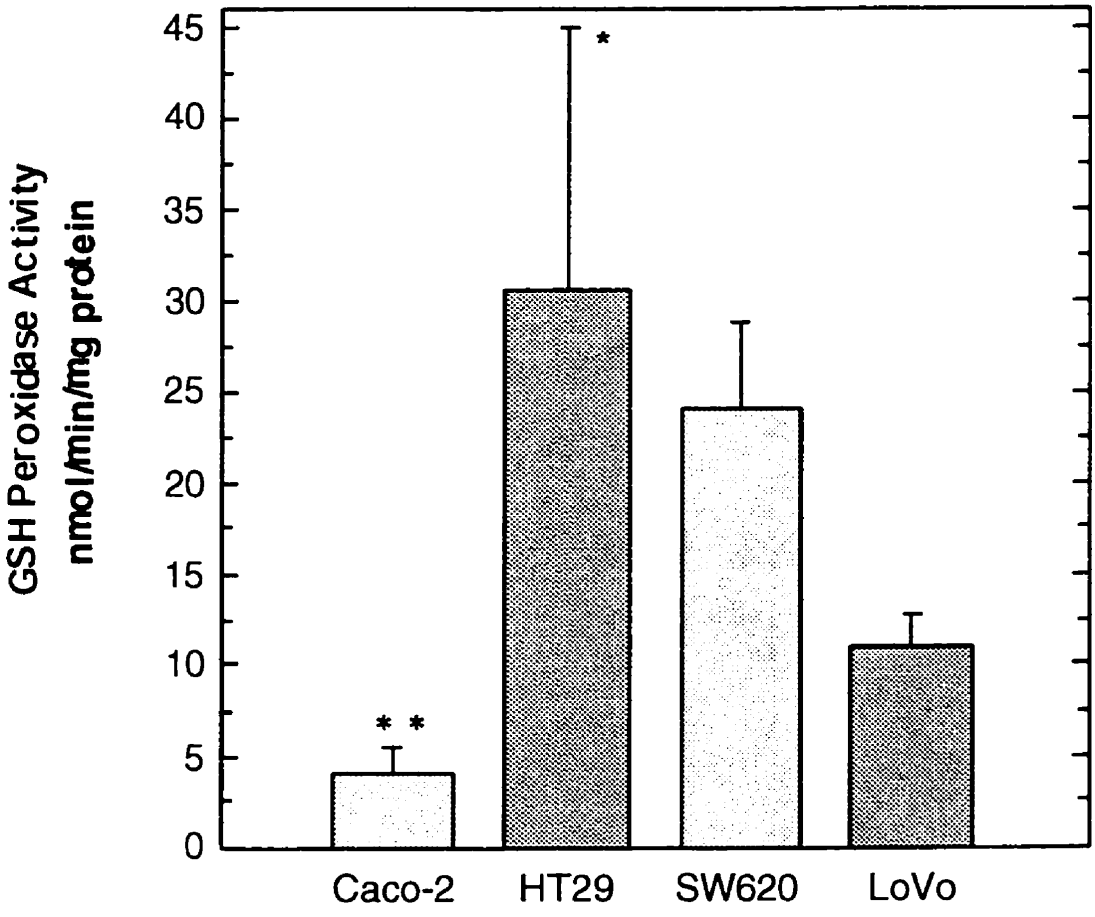

Figure 26: Peroxidation of CHP in cytosol from colon cancer cell lines

Enzyme catalyzed activity toward CHP substrate. Bars represent the mean ± SD of N=3 independent samples, assayed in triplicate for each cell line. Asterisk [*]: One way ANOVA followed by post-hoc Student-Newman-Keuls test detected a significant difference between HT-29 and Caco-2 cytosols ($P<0.01$); [**]significant difference between Caco-2 and all other cytosols ($P<0.05$)

6.4.4 GST Mu Selective Activity

Using the method of Habig et al (1974) the extent of GSH conjugation attributable to GST Mu was assessed using tPBO as substrate. The low rates of conjugation seen in Figure 27 are in agreement with the absence of detectable signals for GST Mu protein and GST M1, M2 and M3 mRNA. The extent of variation among the limited activity occurring was not considered significant (one-way ANOVA).

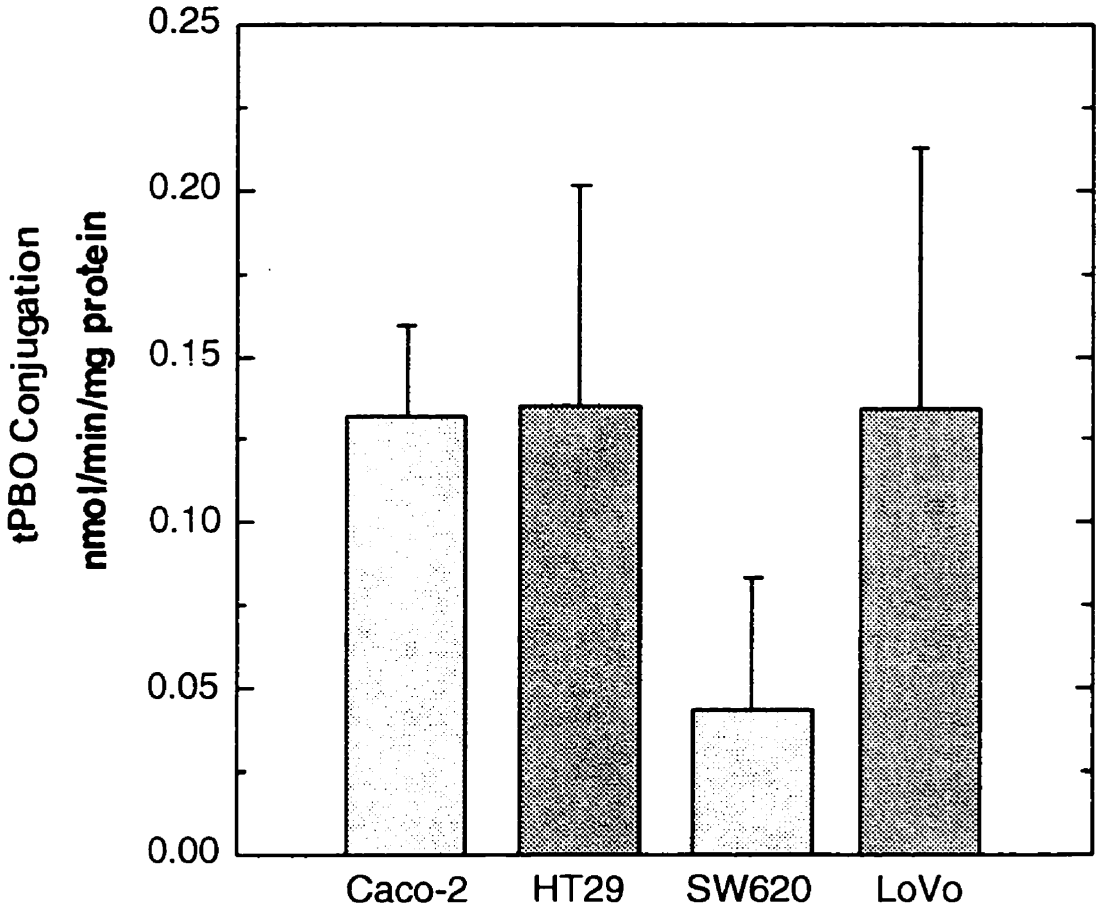

Figure 27: tPBO conjugation as a marker for GST Mu activity

tPBO is used as substrate to measure conjugation by GST Mu. Bars represent mean ± SD of N=3 independent samples assayed in triplicate for each cell line. No significant difference among cell lines was detected by one-way ANOVA (P=0.22).

6.4.5 Catalytic Activity Inhibition

6.4.5.1 Contribution of GST Pi to CDNB conjugation

The CDNB assay was repeated using both Caco-2 and HT-29 cytosols in the presence of Terrapin GST class selective inhibitors to assess their inhibitory potency. TER 117 (see Table 1) is the analogue of GSH reported to have inhibitory capabilities selective for recombinant GST P1 enzyme (Ki = 0.4 μM) (Morgan et al., 1996). Our aim in this section was to assess the ability of TER 117 to inhibit CDNB conjugative activity associated with the GSTs present in colon cancer cell cytosol.

In contrast to the standard CDNB conjugation assay which employed 2 mM GSH, studies performed here employed a greatly decreased GSH concentration. The rationale for the change being, that since the Terrapin inhibitors are GSH analogues which compete for the GSH binding site on the GST enzyme, the ability for them to compete would be hindered by the large amount of GSH contributed by the cytosol, plus that which is added to the reaction cuvette under the existing protocol. Hence, continually decreasing GSH concentrations were assayed, utilizing HT-29 cytosol, to determine a concentration of GSH that would allow reliable measurement of CDNB conjugation and modulation by inhibitors (data not shown). A concentration of 0.05 mM GSH was selected as the amount that would be added to the reaction mixture in addition to that already present in the cytosol. As can be seen in Table 6, addition of 50.0 μM TER 117 to the reaction mixture was able to almost completely inhibit all enzyme-catalyzed conjugative activity in both HT-29 and Caco-2 cytosol. This concentration represents an equivalent amount of exogenous GSH and GSH analogue in the reaction cuvette.

		TER 117		TER 143	
Cell	Dose (uM)	Activity nmol/min/mg protein	Relative Activity	Activity nmol/min/mg protein	Relative Activity
HT-29	0	48.17	1.00	46.91	1.00
	50	0.88	0.02	3.54	0.08
	5.0	5.87	0.12	18.68	0.40
	0.5	31.82	0.66	34.44	0.73
	0.05	49.23	1.02	42.78	0.91
	0.005	49.15	1.02	45.06	0.96
Caco-2	0	45.36	1.00	48.69	1.00
	50	2.38	0.05	2.53	0.05
	5.0	11.47	0.25	17.06	0.35
	0.5	40.02	0.88	36.80	0.76
	0.05	47.80	1.05	43.65	0.90
	0.005	45.03	0.99	44.83	0.92

Table 6: Effect of GST Pi and Alpha inhibitors on CDNB conjugation in HT-29 and Caco-2 cytosol

CDNB conjugation is used as an indicator of total GST conjugation activity. The ability of the free acid forms of the GSH analogues TER 117 and TER 143 to inhibit GST activity is compared. Data are expressed as means of duplicate determinations on a single cytosol preparation.

6.4.5.2 Contribution of GST Alpha to CDNB conjugation

Similar to the previous section, the ability of the TER 143, a GST Alpha selective inhibitor (K_i A1-1=0.27, P1-1=1.9 μM towards recombinant enzyme), was assessed for its effects on CDNB conjugation activity. Again, total GST activity was assessed using HT-29 and Caco-2 cytosol, with the addition of 0.05 mM GSH to the reaction cuvette. As seen in Table 6, TER 143 inhibited total CDNB conjugative activity in a similar pattern to that of the GST Pi selective inhibitor TER 117.

6.4.5.3 Inhibition of GSH peroxidase activity by class selective inhibitors

Using CHP again, as a substrate for GSH peroxidase activity, the ability of TER 117, TER 143 and TER 211 to inhibit catalytic activity was assessed. Despite using concentrations of the GST inhibitors known to almost completely inhibit total GST

conjugation activity, none of the inhibitors were able to produce any appreciable dose-dependent inhibition of CHP peroxidation (Table 7).

		TER 117	TER 143	TER 211
Cell	Dose (μM)	Activity nmol/min/mg protein	Activity nmol/min/mg protein	Activity nmol/min/mg protein
LoVo	0	6.65	6.65	6.65
	50	5.96	4.92	5.12
	5.0	4.92	5.56	6.84
	0.5	4.92	5.76	7.29
	0.05	4.92	5.12	5.37

Table 7: Effect of TER 117, 143 and 211 on GSH peroxidase activity in LoVo cytosol

Using CHP peroxidation as a putative marker for GST Alpha class selective activity, the effect of three GST inhibitors was assessed. Data are expressed as means of triplicate determinations on a single cytosol preparation

6.4.5.4 Inhibition of GST activity by TER 199 in intact cultured cells

After confirming that the GST Pi inhibitor TER 117 (the active, free acid version of TER 199), was capable of inhibiting GSH conjugation in colon cell cytosol, the basis for the lack of potentiation observed for Dox cytotoxicity in our cell culture assays needed to be determined. As others have shown that TER 199 does penetrate the cell membrane and that the diethyl ester is cleaved, what remained to be determined was whether or not the cleaved compounds could inhibit GST in a cellular system. Our approach to answer this question was to incubate a large number of exponentially growing cells with TER 199 for 4 hours (the same period as the MTT assay pre-incubation) and then rapidly harvest the cytosol and assess its total GST activity. Briefly, two 500 cm^2 tissue culture plates of HT-29 cells were cultured with TER 199 in the medium at a concentration of 100, 10 and 1 μM, and control (DMSO). At the end of the incubation period, a rapid cytosol preparation was performed using our standard protocol. The volume of homogenization buffer used was the absolute

minimum that was practically possible (3 ml) so as to limit the extent of dilution of the TER 199. Despite these efforts, it is estimated that the inhibitor was diluted approximately 100 fold from initial concentration during the process of cytosol preparation. Under these conditions, the CDNB conjugation assay did not detect any GST inhibition (Table 8).

		TER 199 (in culture)		
Cell	Dose (μM)	Activity nmol/min/mg protein	St. dev. +/-	Relative Activity
HT-29	0	38.35	1.05	1
	100	38.35	0.90	1
	10	34.55	0.27	0.90
	1	37.41	0.68	0.98

Table 8: Effect of GST Pi Inhibitor TER 199 on the catalytic activity of cells in culture

TER 199 was administered to HT-29 cells cultured in 500 cm^2 plates at 3 concentrations in log increments, for 4 hours. Rapid cytosol preparation was performed and CDNB conjugation activity assay conducted. Values represent mean ± SD of triplicate determinations in a single experiment

6.5 Pgp levels

Each of the cell lines was assessed for expression of the MDR producing, drug exporter, P-glycoprotein. The data in Table 9 show the expression of Pgp in our cell lines relative to those for cells regarded as having high and low expression of the protein. These values clearly show that Pgp is not a contributing factor to the degree of intrinsic drug resistance displayed by these colon cancer cell lines

Cell Line	Pgp Reading	Relative Expression
CEM WT	4	0
CEM 0.1	100	+96
LoVo	10	+6
SW620	5	+1
HT-29	5	+1
Caco-2	3	-1

Table 9: Pgp expression in colon cancer cell lines

Quantitative immunofluorescence image cytometry with MRK-16 Pgp specific antibody. CEM 0.1 used as positive control, CEM wt used as minimally expressing parental line. Values are in arbitrary units from a single assay.

7. Discussion

7.1 Summary of Research Findings

In the interest of developing new chemotherapeutic regimens to combat the intrinsic and acquired drug resistance seen in colorectal cancer, we sought to investigate the involvement of GSTs in this malignancy, and the means by which their contributions can be modulated. Four independent human colon cancer cell lines were cultured and characterized in terms of their GST content and Dox resistance profiles. Additionally, the levels of the MRP and Pgp drug transporters were determined and their contributions to Dox resistance in these cell lines was assessed. The colon cell lines proved to have a broad range of drug sensitivities to Dox, possessing IC_{50}s of 0.12 to 75 μM. All cell lines contained the GST P1 transcript and were immunoreactive to GST Pi polyclonal antibody. The levels of mRNA and protein, however, did not vary significantly between sensitive and resistant lines. None of the cell lines expressed detectable levels of GST Mu transcript or protein and only one of them showed any evidence of GST Alpha expression. The Caco-2 cell line produced a strong signal to GST Alpha polyclonal antibody by western blot, however, the identity of the specific GST protein(s) remains to be determined. Northern blot analysis revealed that of the A1 and A2 GST transcripts probed for, only GST A2 mRNA was detectable. Catalytic activity assays confirmed the presence of functional GST Pi in each of the cell lines. Total GST activity was similar in three lines and significantly higher in Caco-2. The specific GST class contributing to this greater activity in Caco-2 cells could not be ascertained, as

none of the class-selective substrate assays showed any significantly greater activity in this line. Although not statistically significant, a trend was observed in which the two cell lines most resistant to Dox showed the highest levels of GST expression and activity. Caco-2 and HT-29 were consistently the highest and second highest ranking cell lines respectively, in terms of GST Pi mRNA and protein content, total and GST Pi selective conjugative activity and importantly, degree of Dox resistance. Additionally, Caco-2, the most resistant line, possessed a unique expression of GST Alpha. The MRP protein was not detected in any cell line and Pgp levels were extremely low and did not appear to be related to Dox sensitivity. Using both the classic GST inhibiting agent EA, and two novel GSH analogues from Terrapin Technologies (South San Francisco, CA), we were able to successfully modulate total GST conjugative activity in a cell free system. The combination of Dox with a GST inhibitor, however, did not potentiate the cytotoxic effects of Dox in these colon cancer cell lines.

7.2 Cell Growth Patterns in Relation to Resistance

It has been observed that malignancies which possess a faster rate of cell replication are often more sensitive to chemotherapy than slower, more quiescent ones. Most effective antineoplastics rely on changes in the DNA associated with replication in order to inflict their toxic insult. Hence, cells with a higher growth fraction are often more sensitive to antineoplastic therapy. The growth rates of these four cell lines parallel the rank order of sensitivities to Dox, and are more sensitive to Dox when the drug is present for an extended

period of time (several doubling times compared to 4 hours). However, it is unlikely that the relatively small differences in doubling time among the cell lines can account for the 100- to 1000-fold variation in Dox sensitivity observed.

7.3 MTT vs. Clonogenic Assays

The use of the MTT colourimetric microculture assay to investigate the cytotoxic properties of antineoplastics, GST inhibitors and combinations thereof, proved to have advantages and disadvantages, in comparison to the more traditional colony forming assay. After completing an extensive preliminary workup, the MTT assay provided a means of rapidly screening each cell line for the effects of a variety of drug - GST inhibitor combinations. The use of multi-lane and repeating pipettes in cell culture and, automated colourimetry allowed us to conduct a greater quantity of assays with a much higher number of replicates, leading to both smaller experimental error and greater inter-experimental reproducibility. The major disadvantage of the assay appears to be its limited range of detection, especially at the lower end of cell survival. Clonogenic assays did confirm the lack of potentiation of Dox cytotoxicity by EA and Terrapin GST inhibitors observed in the MTT assay. However, the MTT assay was unable to detect the slight potentiation of Chlor toxicity by TER 199 observed in the clonogenic assay. It should be noted that in neither assay were the effects of GST inhibition nearly as great as those observed by other investigators, using similar experimental procedures (Tew et al., 1988; Morgan et al., 1996).

An interesting observation regarding the MTT and clonogenic assays was the opposing responses displayed by the antineoplastics and the Terrapin GST inhibitors. Whereas the cytotoxic potency of both Chlor and Dox was approximately an order of magnitude higher in the clonogenic assay versus the MTT assay, the Terrapin GST inhibitors were much less toxic when evaluated by the clonogenic assay. The simple explanation for the differences observed for the anti-cancer agents may be that the colony forming assay is a more sensitive indicator of a cell's ability to replicate. Even though our MTT protocol employs a 96 hour growth period following drug exposure, the clonogenic assay which provides 14 days for cell growth, clearly provides a more absolute indication of cell survival and cloning efficiency. Comparison of MTT assays performed over three, four or five days reveals that the apparent cytotoxic potency of Dox increases with increasing assay duration. Why then do TER 199 and 143 show lower toxicity in the clonogenic assay versus the MTT assay? One factor may relate to the extent of drug removal that can be accomplished in the two assays. In order to prevent dislodging of the growing cells from the bottom of the wells of the microculture plate in an MTT assay, aspiration of growth medium was never complete, nor was a washing step employed at any time during the assay. The net effect of this is that a certain fraction of drug (GST inhibitor) remains in the culture medium for the duration of the MTT assay, even in experiments denoted as 'acute' exposure. The extensive series of washings and dilutions required as part of the colony forming assay, however, undoubtedly removes most drug (chemotherapeutic

or GST inhibitor) which is not sequestered intracellularly. Additionally, while the majority of Dox is likely intercalated with DNA, the Terrapin compounds may be free to diffuse and may even be extruded from the cell during the washing/dilution steps. To what extent these kinetic factors contribute to the differential toxicity of the GST inhibitors between assays is not known. A second factor may relate to the mechanism by which the Terrapin compounds cause cytotoxicity. Although the mechanisms of cytotoxicity of the GST inhibitors are not well understood, it may be that these compounds interfere with mitochondrial function at higher concentrations. Since production of formazan crystals from MTT depends at least partially on the function of mitochondrial succinate dehydrogenase, it may be that the GST inhibitors interfere with MTT metabolism without causing effects on clonogenicity.

7.4 GSTs and Drug Resistance

Based on the known up-regulation of GST Pi in colon tumours, combined with the reports of GST inhibitors being able to potentiate the cytotoxic effects of antineoplastics in both cancer cell lines and xenografts, we assayed the GST Pi protein and mRNA levels in four colon cancer cell lines to determine if GST expression was related to the degree of drug resistance. The data clearly show that while the enzyme is present in all four cell lines, the variation is insufficient to account for the large range of drug sensitivities observed. In addition, catalytic activity assays designed to determine the contribution of the GST Pi protein to the overall GSH conjugation, did not

provide evidence for this enzyme as a major factor in producing the range of sensitivities observed.

Protein, mRNA and catalytic activity assays showed consistently that none of the GST Mu enzymes were expressed to any appreciable level in these colon cell lines. This is not entirely unexpected as GST Mu is infrequently expressed in colon (Campbell et al., 1991; Tew et al., 1996). Moreover, expression is often decreased in tumour compared to normal tissues (Ranganathan and Tew, 1991).

Assays for GST Alpha content and activity have produced some interesting results. The unique protein expression and the positive GST A2 mRNA northern blot for Caco-2 cells have provided the only suggestive evidence in our study that GST may be involved in resistance of this cell line to Dox. This expression of GST Alpha in Caco-2 cells has previously been observed by others and is ascribed to be a cause of this cell lines resistance to Dox (Peters and roelofs, 1992). These reports of protein expression are, however, in contrast with the results of our catalytic activity assays which show this cell line to have the lowest peroxidase activity of the four cell lines. It is possible that what is being measured under the CHP protocol is not the peroxidase activity of GST Alpha but perhaps the activity of the selenium-dependent glutathione peroxidase (GPX) enzyme. We have not assessed by immunoblot or northern blot for the presence of GPX, however, two key lines of evidence lead us to this hypothesis. Firstly, upon repeating the assay with the substitution of hydrogen peroxide (H_2O_2) for CHP, it was observed that the extent

and rank order of peroxidase activity were nearly identical to the original experiment (data not shown). The relevance of this finding is that inorganic peroxides such as H_2O_2 are not known to be acted upon by any class of GST. Secondly, the GST Alpha selective inhibitor TER 143 was ineffective (at concentrations up to 500 μM) at inhibiting the peroxidase activity while using CHP as substrate. Hence, what we believe is being measured here is predominantly GPX activity. These results do not preclude that any GST Alpha catalyzed peroxidation is occurring, however, the extent may not be sufficient to be observed in the presence of GPX.

While GST Pi is clearly the most predominantly expressed enzyme, both in terms of concentration and frequency of expression, there is some support for the Alpha class of GSTs being contributors to Dox resistance. GST A1-1, A1-2 and GSTA2-2 have all been shown previously to possess significant peroxidase activity (Hayes and Pulford, 1996). Although the extent of GST mediated activity may be masked by GPX in our study on CHP, a contribution from GST Alpha enzymes in addition to GPX in the oxidatively-stressed cell may provide the extra degree of protection necessary to prevent free radical based lipid peroxidation from reaching a lethal level. In addition to the GST Alpha classes examined in our study, there are a number of other not yet fully characterized isoforms which may be active in the colon. Of note, there is evidence to indicate the involvement of the human homologue of mouse GSTA4-4, hGST 5.8, as a lipid peroxidation defense mechanism (Singhal et al., 1994). This recently identified GST is known to possess activity toward CDNB

(which would be detected in our total GST activity assay) but has not been found to be active toward the agent we used to assess peroxidase activity (CHP) (Hayes and Pulford, 1996). If this or a similar GST is over expressed in Caco-2 cells, it might explain why this cell line displays the highest CDNB conjugation but low CHP peroxidation. While not being active toward CHP, hGST 5.8 possesses significant activity toward the highly reactive and potentially genotoxic products of lipid peroxidation, such as 4-hydroxynonenal, which may be a more accurate indicator of effective cellular defense than the non-physiologic substrate CHP. Additionally, transfection of mGSTA4-4 into Chinese hamster ovary cells (CHO) has been shown to produce a 40% reduction in lipid peroxidation subsequent to Dox exposure and a concurrent 2-fold higher resistance to this drug (He et al., 1996). hGST 5.8 (based on its pI) is structurally homologous to the Alpha class of enzymes, and is recognized by anti-recombinant mGSTA4-4 antibodies; it is, however, not recognized by antibodies to any of the human Alpha, Mu or Pi proteins, which makes it unlikely that this protein is the same as the one detected in Caco-2 cells. A GST A3 enzyme has also been identified in humans, but this enzyme is not yet functionally characterized. It is not known if any of our polyclonal antibodies can detect this GST protein.

Using an alternate approach to both detect and modulate GST Alpha activity, the GST Alpha selective inhibitor (TER 143) was used in a standard CDNB conjugation assay on two colon cell lines. We predicted that Caco-2 cells, which express at least one member of the GST Alpha class, would be

more sensitive than HT-29 cells to the inhibitory effect of TER 143. Instead we observed an equal degree of inhibition in cytosol prepared from both lines. This lack of differential inhibition may indicate a number of possible situations. Firstly, it may indicate that a similar GST enzyme activity profile is being inhibited in both cell types. That is, the Alpha class protein detected in Caco-2 cells may not be active toward CDNB, and similar amounts of functional GST Pi enzyme are present. Secondly, as discussed for GSH peroxidase activity, it may be that the degree of Alpha activity is greatly overshadowed by that of the GST Pi and/or GPX enzymes. A third possibility may be that TER 143, which has been developed based on selectivity for the GSTA1-1 recombinant enzyme, is ineffective against the GST A2-2 or other Alpha enzymes that are present in Caco-2 cells.

The ability of TER 117 to inhibit GST Pi activity was confirmed using HT-29 and Caco-2 cytosols. Subsequently, the ability of the TER 117 diethyl ester (TER 199) to enter cells in culture, be cleaved to its active form, and then inhibit GST, was assessed. Despite alterations in our standard protocols geared towards magnifying the effects of GST inhibition, we were unable to detect any such activity. As others have provided evidence that TER 199 does indeed enter the cell (Morgan et al., 1996), it is speculated that the lack of observed effects is due primarily to the diluting effects of the sample preparation (approximately 100-fold). Thus, in order to detect inhibition by this assay, we estimate that an excess of 1.0 mM of the compound would have to be applied in culture. This higher dose of compound was not used for two main reasons:

1) evidence of altered cell viability was already visible at the time of harvest for cultures treated with 100 μM TER 199 and; 2) since the test dose would be 200-500 fold above that which could be used in vivo (based on the IC_{50}s determined in our study), assessment at this level would be clinically irrelevant.

Comparing the results of our ***in vitro*** CDNB conjugation activity inhibition by TER 117 and TER 143, it is intriguing that the extent of inhibition by TER 143 (GST Alpha selective inhibitor) is nearly identical to that produced by TER 117 (GST Pi selective inhibitor), despite a reported 5-fold lower K_i of the later agent toward recombinant Pi enzyme (Morgan et al., 1996). If GST Pi is indeed the predominant GST enzyme present in the colon cell lines, we expected TER 117 to be a more potent inhibitor of CDNB conjugation than TER 143. Clearly, the enzymatic complexity of cytosolic preparations makes direct extrapolation from studies performed on purified recombinant GSTs very difficult.

7.5 MRP Findings

Immunoblot analysis of the 106,000 x g membrane fraction of our four colon cancer cell lines did not detect any MRP expression. Based on findings by others who have assayed for MRP in colon cell lines, this lack of expression is not surprising, although, there are some technical differences that should be noted. Firstly, our studies are most similar to those of Flens et al (1996) who used a panel of tissues which were individually homogenized and used to prepare a membrane fraction for immunoblot analysis. This study found 15 of 16 colon tumours to be negative for MRP protein expression. Several papers, on the other hand, have reported expression of MRP in 42% to 100% of colon

samples. These studies employed either mRNA transcript or immunohistochemical analyses to reach their conclusions (Kruh et al., 1995; Chuman et al., 1996; Endo et al., 1996; Kuo et al., 1996). As the above studies were conducted both in human tumour samples and also cultured cell lines, one can exclude the possibility that expression is significantly altered in the maintenance of a cultured line. It could, however, be speculated that the membrane preparation procedures employed by us and the Flens group were not ideal for harvesting and preserving the MRP protein. Additionally, those studies which did show positive expression of MRP protein in the colon used a different monoclonal antibody to MRP than the one used both by us and the Flens group. As mentioned earlier, the 95 kDa band observed by both our groups on immunoblot has been attributed to direct binding of secondary antibody. The large amount of protein electrophoresed and transferred to the membrane makes this type of extraneous band not unexpected. It should be noted though, that the Flens group observed this band while using a rabbit-anti-mouse immunoglobulin, whereas our study found the band with a donkey-anti-mouse immunoglobulin.

These technical concerns and discrepancies in MRP expression make it difficult to draw conclusions regarding the contribution of this drug transporter to intrinsic drug resistance in colon cancer. Based strictly on the findings of our immunoblot analysis, it does not appear that MRP is responsible for the range of toxicities seen in these cells toward Dox.

While the MRP transporter may not have been identified as a contributor to the range of sensitivities seen in these colon cell lines, there are other drug transporters which could fill this role. Some GSH conjugate transporters being investigated include variants of the canalicular multispecific organic anion transporter (cMOAT) (Muller et al., 1996), the lung resistance-related protein (LRP) (Scheper et al., 1993) and an interesting but sparsely studied protein known as dinitrophenyl-S-glutathione (DNP-SG) ATPase (Awasthi et al., 1994b; Muller et al., 1996). This latter protein has been isolated from lung and erythrocytes and has a molecular weight of approximately 38 kDa, which is much smaller than the other known transporters. Transport is ATP-dependent, competitive with GSH conjugates and other prototypical Pgp substrates and importantly, studies on inside-out vesicles show active uptake of Dox without any requirement for GSH conjugation or other modifications. It is anticipated that future studies on this and other transporters of GSH conjugates and natural product antineoplastics will assess their role in drug resistance.

7.6 Pgp Findings

The low levels of expression and lack of correlation between Pgp and drug sensitivity suggest that this resistance mediator is not a factor in producing the range of sensitivities displayed by our four colon cancer cell lines. The data are in agreement with other studies which indicate minimal or no expression in HT-29, Caco-2 and many other non-drug-selected colon cell lines (Kramer et al., 1993). Recently, the expression of Pgp in Caco-2 cells has

been investigated by others and found to be dependent on the state of cell growth and differentiation (Hosoya et al., 1996).

Our findings on intrinsic Dox resistance do not, however, preclude the involvement of Pgp in ***acquired*** drug resistance. For example, SW620 is reported to intrinsically expresses only low levels of Pgp (as evidenced here), but is known to express very high levels of the drug transporter in Dox-selected sub-lines (Lai et al., 1991; Rabier et al., 1991).

7.7 Topo II

As direct inhibition of topoisomerase II activity is generally accepted as the primary mechanism of Dox toxicity, and variations in this enzyme's level and activity are reported to generate an atypical MDR (Hoban et al., 1992), assessment for relative levels of this enzyme in these colon cells would be an excellent addition to the resistance profile generated by this study. This will be an important direction for future research.

7.8 GST Inhibition and Potentiation of Dox Cytotoxicity

The use of GSH analogue-based inhibitors as adjuncts to chemotherapy does not appear to be an effective means of potentiating the Dox cytotoxicity in intrinsically resistant colon cancer cell lines. The four cell lines investigated here displayed no consistent enhancement of Dox toxicity beyond the additive effects of the antineoplastic and the GST inhibitor. This lack of potentiation, as assessed by MTT assay, was confirmed in the clonogenic assay at least for a single, moderately resistant cell line. Despite the shortcomings of the MTT assay, in terms of the limited range of detectable cytotoxicity, we are confident

that the additive toxicity that was seen in the colony formation assay for HT-29 cells, is representative of the effects we are likely to see in the remaining cell lines, based on the similarity of their GST content and activity profiles. As these GSH analogues have been shown by others to significantly potentiate the effects of certain antineoplastics, it remains to be determined whether the effects observed here are due to a failure of the drug to effectively inhibit GST in intact cells or whether the importance of GSTs in the protection of these colon cell lines against Dox has been overestimated. Very recent findings by other investigators who have successfully enhanced the effects of Dox by employing antisense technology (in order to decrease intracellular GST levels), have reinforced the concept that GSTs, specifically the Pi class, may be involved in drug resistance (Ban et al., 1996).

It is now firmly established that antineoplastic drug resistance is often multifactorial. Variation in GST activity is one factor that may contribute to a drug-resistance phenotype, depending on the type of cell and antineoplastic involved. Future work should focus on assessment of Topo II, GSH status, and selenium-dependent GPX as potential resistance mediators in human colon cancer. This study has evaluated four colon cancer cell lines, for which selection was based on previously reported sensitivities to Dox. Perhaps a study which investigated cell lines chosen for their known variations in GST levels instead, would provide a better indicator of what role GST modulation could play in enhancing the effects of chemotherapy. There may be a certain critical level of GST expression, above or below which pharmacologic

manipulation of GST is not effective. Future studies in this area may benefit from absolute quantitation of GST enzymes in the cell lines as opposed to the relative quantitative techniques employed here.

Ongoing studies on pharmacologic GST modulation will likely focus on alkylating agents and other antineoplastics which are known to be detoxified by direct conjugation to GSH. Evidence of potentiation in a larger number of cell lines will likely be required prior to clinical trials, as will safety and efficacy studies. Investigators at Terrapin Technologies, the manufacturers of the GST inhibitor compounds used in this study, have recently heightened the level of drug targeting to cancer cells by developing a latent alkylating agent activated by the GSTs which are known to be over-expressed in a number of cancers (Satyam et al., 1996).

8. Conclusions

The findings of this study lead us to conclude that GST Pi content and activity are not related to intrinsic Dox resistance in colon cancer cell lines. However, further evaluation of the contribution of GST Alpha to Dox resistance is warrented in cell lines like Caco-2 that appear to overexpress members of this class. Assessment of the levels of MRP and Pgp indicate that these drug transporters are not involved in intrinsic Dox resistance, despite reports of their extensive involvement in Dox resistance-selected sub-lines of some of the colon cancer cell lines studied here. Based on this assessment, we are not able to provide a specific explanation for the range of sensitivities observed, other than a hypothesis that intrinsic Dox resistance is multifactorial and is mediated by one or more of the non-MDR resistance mechanisms described earlier. Our MTT cytotoxicity assay provided us with a relatively rapid means of determining cellular responses to chemotherapy, however, its limited range of detection renders it less than ideal for elucidating relatively subtle potentiation effects of GST inhibitors that may occur preferentially under conditions that yield a high degree of cell kill. Lastly, pharmacologic manipulation of GST activity in non-drug-selected colon cancer cells does not appear to have a specific impact on the toxicity of the anthracycline doxorubicin, despite evidence of potentiation effects on other classes of antineoplastics.

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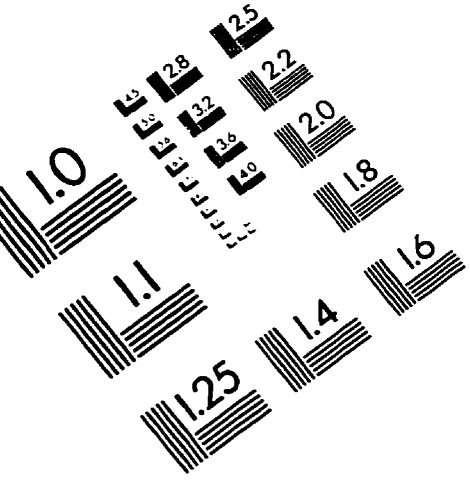
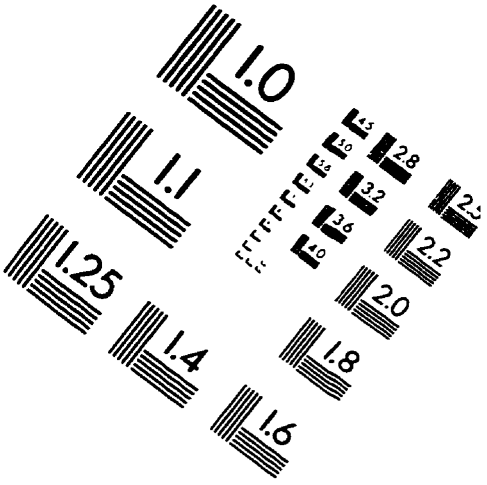
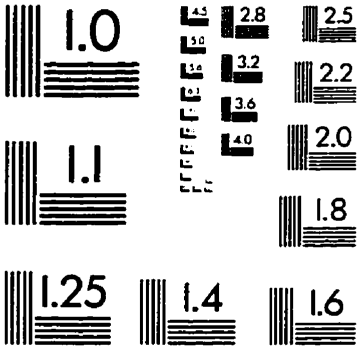
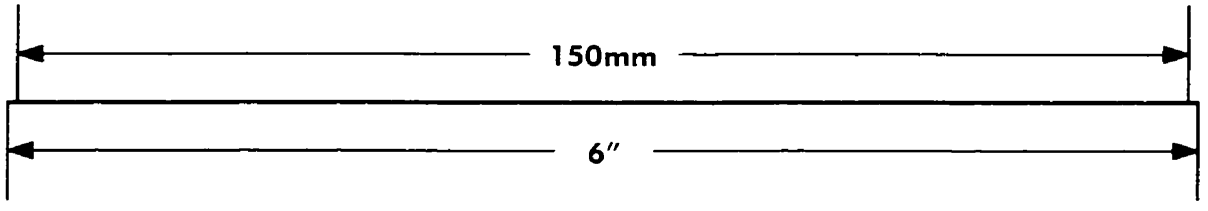
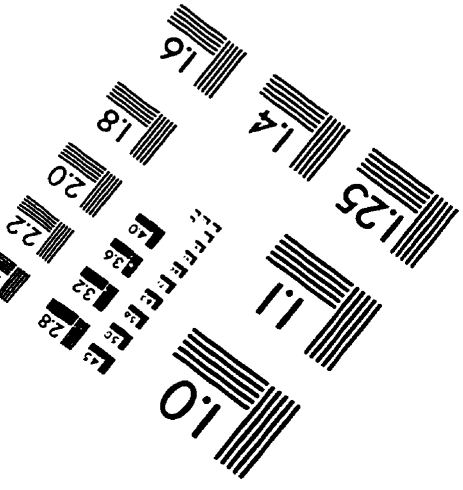
IMAGE EVALUATION TEST TARGET (QA-3)

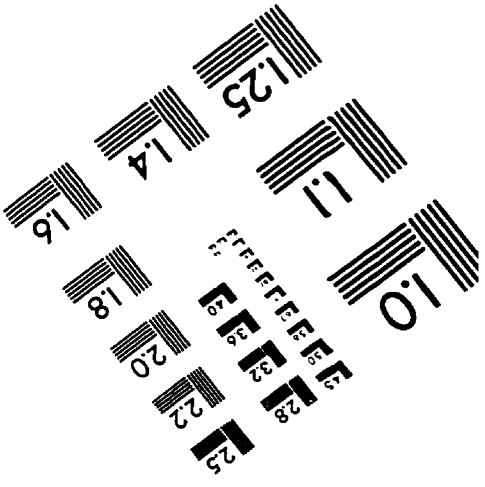












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