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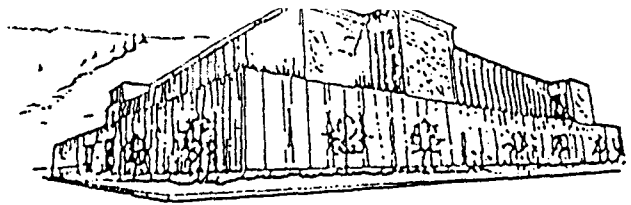
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# COLCHICINE-INDUCED MULTIPLE DRUG RESISTANCE IN C-6 GLIOMA

By

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B.S., Medical Technology, University of Montana, 1970

Presented in partial fulfillment of the requirements for the

Degree of Master of Science

University of Montana

1994

Approved by:



Chairman, Board of Examiners



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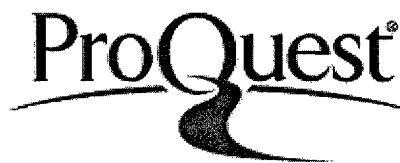
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James D. Hutchison, Jr., M.S., 1994 Pharmacy

Colchicine Induced Multiple Drug Resistance In C-6 Glioma (80pp.)

Director: Charles L. Eyer, Ph.D. 

Multiple drug resistance (MDR) is a common problem that occurs with repeated exposure to some anticancer drugs. MDR is produced by increased synthesis of an active transport system that lowers intracellular levels of a variety of anticancer drugs. Colchicine has been shown to induce MDR in several cancer cell lines. Cancer treatment with taxol has been complicated by the the induction of MDR with repeated exposure. C6 glioma represents a well characterized model of the most common human brain tumor. We investigated the ability of colchicine to induce resistance to taxol in this cell line. Our research focused on three objectives: a). to determine whether drug resistance to taxol can be induced by colchicine in a C6 glioma cell line. b). to determine colchicine-induced changes in protein content, stress response, and key enzyme levels (heme oxygenase (HO), glutamine synthetase (GS), and cyclic nucleotide phosphohydrolase (CNP). c). to determine whether drug resistance can be reversed by verapamil -- a known inhibitor of the multiple drug resistance transport system.

Drug resistance was verified by comparing the EC<sub>50</sub> values of both the control and colchicine treated cells upon exposure to increasing concentrations of taxol. Colchicine applied for a 24 hour period at a concentration of 480 ng/ml induced drug resistance to taxol in the C-6 glioma cell line, as evidenced by a greater than 14 fold increase in taxol's EC<sub>50</sub>. The resistant strain also exhibited is an increase in the amount of protein per cell; and increased HO activities. Drug resistance was reversed by verapamil. These studies show that colchicine has induced resistance to taxol and that the resistance appears to be related to the induction of the multiple drug resistance receptor.

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# INTRODUCTION

## a. Drug Resistance:

Oncologists agree that one of the important unsolved problems in cancer treatment is drug resistance. This includes both intrinsic resistance at the time of initial chemotherapy and acquired drug resistance. In general terms, acquired drug resistance may result from alterations in host drug metabolism, from spread of tumor cells to sites poorly accessible to chemotherapy (“sanctuary sites”), and/or from biochemical changes at the cellular or subcellular level (Perez *et. al.*, 1993). (TABLE 1.)

***Table 1. Mechanisms Of Resistance To Chemotherapy***

Host factors
Altered pharmacokinetics
Decreased drug absorption-activation
Enhanced drug excretion-degradation
Altered binding-transport proteins
Host-tumor factors
Metastasis of cells to “sanctuary sites”
Cellular factors
Decreased drug accumulation
Decreased influx or increased efflux
Altered cellular metabolism
Decreased activation or increase degradation
Cytoplasmic-nuclear inactivation
Glutathione / Metallothioneins / ? proteins
DNA repair / tolerance to DNA damage
Altered cellular target

To investigate the basis of drug resistance, drug-resistant cell lines have been isolated by exposing various cancer cell lines to increasing amounts of chemotherapeutic

antibiotics, for example, adriamycin; the resistant cells that are isolated are frequently not only resistant to adriamycin but may be cross-resistant to most naturally occurring antibiotics. The simultaneous resistance to many different structurally unrelated drugs is called multiple drug resistance (MDR). MDR includes resistance to many natural products isolated from plants (e.g. Taxol, colchicine) and microorganisms. Multiple drug resistance does not appear to extend to agents synthesized in the laboratory such as cisplatin, cytosine arabinoside, cyclophosphamide, and methotrexate (Gottesman and Pastan, 1988). (TABLE 2).

***Table 2. Drugs In The Multiple Drug Resistance Group***

ANTICANCER DRUGS	OTHER DRUGS
ACTINOMYCIN D DAUNOMYCIN DOXORUBICIN ETOPOSIDE (VP-16) MITOXANTRONE TAXOL VINBLASTINE TENIPOSIDE (VM-26) VINCRISTINE	COLCHICINE EMETINE ETHIDIUM BROMIDE GRAMICIDIN D MITHRAMYCIN PUROMYCIN VALINOMYCIN

The drugs in the MDR group do not share a common mechanism of action; some affect microtubules and some inhibit DNA, RNA, or protein synthesis. The biochemical basis of this type of resistance came from studies showing that MDR cells accumulated less drug (Dano, 1973; Ling and Thompson, 1974). Drug entry appeared to be normal, but the cells had acquired the capacity to pump out the drugs (Fojo, *et. al.*, 1985). There

was no good precedent for a single transport protein that could pump such structurally diverse drugs out of cells.

One common feature of the drugs in the MDR group is that they are moderately soluble in both water and lipid (amphipathic). The solution to how a single transport protein or proteins could pump out such structurally diverse drugs from cells came when the gene responsible for MDR (MDR1-also known as p-glycoprotein) was isolated from a very highly drug-resistant cell line derived from a cervical carcinoma (Robinson, *et. al.*, 1986; Chen, *et. al.*, 1986).

With the isolation of a full-length cDNA for the MDR1 gene, it was possible to transfer the MDR1 cDNA into drug-sensitive cells and confer resistance. Because the introduction of a single gene in a drug-sensitive cell led to resistance to adriamycin, vinblastine, actinomycin D, taxol, and colchicine, it was concluded that a single membrane protein must be able to transport a wide variety of drugs that are structurally dissimilar (Pastan, *et. al.*, 1988).

The first step in drug chemotherapy is the transport of the drug from an aqueous extracellular environment into the lipid environment of the plasma membrane. From here the drugs can diffuse into the cytoplasm and nucleus where their cytotoxic action occurs. The drugs may encounter the p-glycoprotein transporter to pump them back out of the cell (Cornwell, *et. al.*, 1986, 1987). Recent data support the idea that the pump recognizes and expels drugs directly from the plasma membrane (Raviv, *et. al.*, 1990).

Many drugs that have no antineoplastic activity are also substrates for the multiple drug transporter and can competitively block the transport of the cytotoxic drugs (Pastan

and Gottesman, 1991). When present in excess, these inhibitors overcome MDR by preventing the multiple drug transporter from removing adriamycin, vinblastine, colchicine or other cytotoxic agents from resistant cells (TABLE 3).

**Table 3. Drugs Currently In Trial To Reverse Multiple Drug Resistance**

Calcium channel blockers R- & L- Verapamil Cyclosporine A and its analogs Hydrophobic Cephalosporins	Phenothiazines Quinidine & Quinine Reserpine Yohimbine
--	--

Dalton and coworkers studied multiple drug resistance expression in multiple myeloma and showed that patients who no longer responded to a regimen including vincristine, adriamycin, and dexamethasone did become responsive when treated with verapamil. Some patients developed a transient remission (Meister, 1985).

A  $^{32}\text{P}$ -cDNA probe to measure MDR1 RNA levels and antibodies was used to determine the function and precise cellular location of the multiple drug transporter (Thiebaut, *et. al.*, 1987; Fojo, *et. al.*, 1987). The transporter has been detected in the liver, kidney, large and small intestines, pancreas, brain, testes, and adrenal cortex. Since the substrates for the multiple drug transporter are natural products found in plants and microorganisms, researchers believe the transporter evolved as a protective mechanism to prevent the entry of toxic compounds from the intestine or to remove toxic compounds via the bile and urine if they were absorbed. In addition, the transporter prevents compounds from entering the brain (perhaps as part of the blood-brain barrier) and the testis (Pastan and Gottesman, 1991).

Colchicine is an alkaloid that can be obtained from two members of the lily family: autumn crocus and glory lily (Ferguson, F.C., 1952). Colchicine binds to tubulin and thus prevents its polymerization into microtubules. This antimitotic property disrupts the spindle apparatus that separates chromosomes during metaphase (Wallace, S.L., *et. al.*, 1970). Colchicine has been used to induce drug resistance and to investigate the properties of multiple drug resistance in human ovarian cancer cells and in transfected mouse cell cultures (Ling, V., *et. al.*, 1974; Podda, *et. al.*, 1992).

Taxol, a taxane alkaloid, is the major active species of extracts derived from the bark of the Pacific yew, *Taxus brevifolia* (Wani, M.C., *et. al.*, 1971). The mechanism of action of taxol has been demonstrated to be unique, constituting tubulin polymerization and stabilization of microtubules rather than the depolymerization reported for the classic antimicrotubule agents vincristine and colchicine (Schiff, P.B., *et. al.*, 1979). Cross resistance of taxol with other natural products has been demonstrated in vitro (Gupta, R., 1985).

Verapamil is one of several drugs currently in clinical trials to reverse the multiple drug resistance associated with cancer chemotherapy (Pastan, I., and Gottesman, M., 1991). The l-stereoisomer, is used clinically as a calcium channel blocker. Verapamil appears to inhibit the efflux of toxic chemotherapeutic drugs by acting as a substrate of P-glycoprotein, thereby, allowing drugs like vincristine, colchicine, and taxol to remain within the cell and exert their cytotoxic action (Racker, E., *et. al.*, 1986).

#### **b. Heat shock proteins / stress response:**

A major question in molecular biology is how cells cope with rapid changes in their environment, such as exposure to elevated temperatures (heat shock), heavy metals,

toxins, oxidants, drugs, bacteria, and viruses. It has become clear that all living organisms share a common molecular response that includes a dramatic change in the pattern of gene expression and the elevated synthesis of a family of heat shock or stress-induced proteins (Morimoto, *et. al.*, 1990; Lindquist, *et. al.*, 1988).

### **i) Background and Significance**

Immediately after a sudden increase in temperature, all cells—from the simplest bacterium to the most highly differentiated neuron—increase production of a certain class of molecules that buffer them from harm. When biologists first observed that phenomenon 30 years ago in *Drosophila* cells (Tissieres, *et. al.*, 1974), they called it the heat shock response. Subsequent studies revealed that the same response takes place when cells are subjected to a wide variety of other environmental assaults, including toxic metals, alcohols (Neuhaus-Steinmetz, *et. al.*, 1993), and many metabolic poisons (Schlesinger, 1990--TABLE 4).

***Table 4. Inducers Of Stress Response Family Members***

Environmental	Pathophysiologic	Other
-Heat	-Microbial infections	-Cell cycle
-Heavy metals	-Tissue trauma	-Embryonic development
-Organics	-Genetic lesions	-Cell differentiation
-Oxidants		-Hormone stimulation
		-Microbial growth

The ability to respond to environmental challenges is a fundamental requirement for the survival of all living organisms. This is consistent with the observation that one of



the mechanisms by which cells respond to such signals (the heat shock/stress response), and many of the proteins that are synthesized as components of this response are highly conserved among widely divergent organisms. For example, the major heat shock protein, Hsp 70, has about 50% of its sequence conserved between *E. coli* and human, and some domains are 96% similar. (Burdon, 1986 and Lindquist, 1986).

The Hsp 70 proteins (heat shock protein—molecular weight approximately 70,000 dalton) are needed for import of several proteins into eukaryotic cell organelles. Other Hsp 70 family members act inside these organelles, as does a protein that belongs to a different family of heat shock proteins, the Hsp 60 or GroEL group. For import into the organelle, it is proposed that Hsp 70 unfolds the partially folded polypeptide so that it can be translocated through a membrane pore (Hightower, 1980). Consistent with this role are data showing that Hsp 70 like protein can bind to and, in the presence of ATP, dissociate protein complexes. Among these are clathrin-coated vesicles, a  $\lambda$  bacteriophage DNA replication complex, nucleolar proteins that have become insoluble as a result of heat shock and immunoglobulin heavy chains formed in the absence of light chains.

In most stressed cells, the newly made Hsp 70 localizes in the nucleus and the nucleolus where it is tightly complexed in an insoluble form that is partially solubilized by ATP. The nucleolus is the site of ribosome assembly and is unusually thermal sensitive. It is suggested that Hsp 70 binds to proteins that are incompletely folded in the preribosome assembly unit and protects them from irreversible denaturation.

Another family of heat shock proteins, Hsp 60's, also form complexes with polypeptides and has ATPase activity. In contrast to the postulated unfolding and disassembly role of most Hsp 70's; Hsp 60's participate in the folding and assembly of polypeptides. Based on this property, they have been referred to as chaperonins (Hemingsen, *et. al.*, 1988). In the eukaryote, the Hsp 60's are localized in cytoplasmic organelles such as mitochondria and chloroplasts.

A third heat shock protein family is the Hsp 90 (approx. 90,000 daltons) group. In the eukaryote Hsp 90 is abundant in normal cells, is highly phosphorylated on serines and threonines, and is localized in the cytoplasmic compartment of the cell. A small fraction of it translocates to the nucleus after heat shock. Like the Hsp families noted above, Hsp 90 complexes with a variety of normal cellular proteins (TABLE 5).

The most thoroughly studied are the glucocorticoid receptors that are maintained in inactive conformation bound to Hsp 90 until activated by the hormones. Several kinases are transiently complexed with this heat shock protein; most notable are the tyrosine kinases encoded by oncogenes. Another kinase, one that phosphorylates the eukaryote translation-initiation factor eIF-2a subunit, is activated by Hsp 90. The cytoskeletal proteins, actin and tubulin, are associated noncovalently with Hsp 90. Hsp 90's may also function as a chaperonin. Microfilaments and microtubules (which are affected by both colchicine and taxol) are not unusually sensitive to stress although a prolonged and severe heat shock modifies both structures. In contrast, the intermediate filament network is

very thermal sensitive (Parcell and Sauer, 1989). It is believed that the Hsp 90 protects and aids in the recovery of these cytoskeletal systems.

**Table 5. Proteins Complexed With HSP**

HSP 70 Family	HSP 90 Family	Hsp 60 (GroEL) Family
<ul style="list-style-type: none"> <li>-clathrin-coated vesicles</li> <li>-prepro <math>\alpha</math> factor</li> <li>-nucleolar proteins</li> <li>-IgG Heavy chain</li> <li>-p53 tumor antigen</li> <li>-DNA replication initiation</li> <li>-complex (phage, plasmid)</li> <li>-calmodulin</li> <li>-SV40 T-antigen</li> <li>-microtubules (<math>\beta</math>-internexin)</li> </ul>	<ul style="list-style-type: none"> <li>-glucocorticoid receptor</li> <li>-tyrosine kinases</li> <li>-eIF-2a kinase</li> <li>-yeast protein kinase C</li> <li>-tubulin</li> <li>-actin</li> </ul>	<ul style="list-style-type: none"> <li>-<math>\lambda</math> phage collars</li> <li>-ribulose-p<sub>2</sub> carboxylase/ oxygenase heavy chain</li> <li>-cytochrome c</li> <li>-F1-ATPase</li> <li>-temperature-sensitive mutants</li> </ul>

In addition to improper polypeptide folding, heat shock leads to a plethora of changes that are dependent on both the intensity of the stress and on the cell system. These include effects on macromolecular synthesis, on levels of cations, on states of protein phosphorylation, on metabolic pathways, and on cytoskeleton networks (Parcell, et. al., 1989).

#### ii) Interaction between MDR and the stress response

Chin, *et. al.*, (1986) reported that exposure of a renal adenocarcinoma cell line to heat shock or sodium arsenite increased MDR1 mRNA levels (Chin, K-V., *et. al.*, 1990). In a separate study in which a hepatocarcinoma cell line HepG2 was treated with sodium

arsenite about a twofold increase of MDR1 mRNA was observed (Kioka, *et. al.*, 1992). Heat shock at 45°C for 10 minutes also increased the level of MDR1 mRNA. Heat shock at 42°C for 1-2 hours, also had a marginal effect.

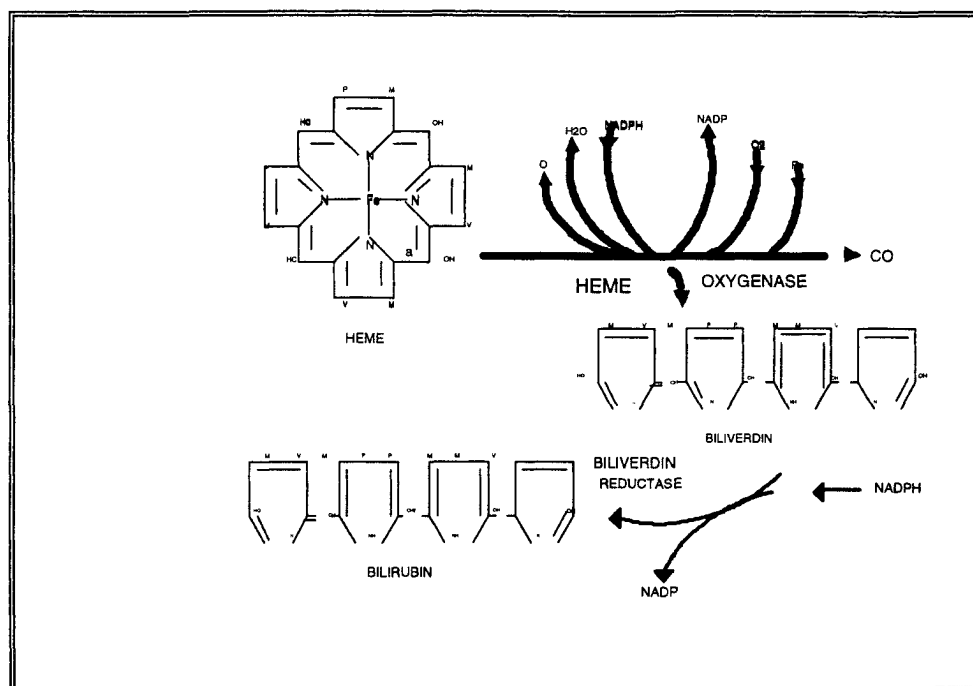
Thermotolerance, resistance to oxidative stress, and induction of stress proteins were examined in a panel of 10 human tumor cell lines (Steels, *et. al.*, 1992). This research showed that a heat shock of 42.5°C for 30 min. was sufficient to induce tolerance to a subsequent heat stress of 43.5°C for 3 hr. in four human cell lines. The initial heat shock itself was not lethal (100% survival). The heat stress alone resulted in 20-40% survival in four of the cell lines. Induction of Hsp 70 was evident in HeLa and two melanoma cell lines two (2) hours after heat shock. However, this treatment did not induce other Hsp's (Hsp 28, Hsp 90).

### **iii) Heme oxygenase**

Heme oxygenase is an enzyme which catalyzes the oxidative conversion of heme (iron-protoporphyrin-IX Fe-heme) to the linear tetrapyrrole biliverdin by insertion of two oxygen atoms into the tetrapyrrole ring adjacent to the alpha-methene bridge (Trakshel, *et. al.*, 1988) (FIGURE 1).

The oxygen atoms utilized in this reaction are derived from two separate molecules of oxygen. An additional molecule of oxygen is used to convert the alpha-methene carbon to carbon monoxide. During this process the central iron atom is released. The microsomal origin of the enzyme was first described by Tenhunen, *et. al.*, in 1968. The substrate and kinetic characteristics of the enzyme in microsomes were defined thereafter (Maines, 1984).

Heme oxygenase has been resolved into two isozymes, designated HO-1 and HO-2, appearing in several tissues including brain (Cruse and Maines, 1988). HO-1 and HO-2 appear to be products of different genes (Trakshel and Maines, 1989). They differ immunologically and physically and show species diversity (Shibahara, *et. al.*, 1987). Similarities have been observed with respect to cofactor requirements for activity, sensitivity to inhibitors, as well as substrate used, i.e., heme, hemohe, and cytochrome c. Both forms of the enzyme require NADPH-cytochrome c (P-450) reductase, NADPH, or NADH, and O<sub>2</sub> for activity. The activity of both forms can be inhibited by potassium cyanide, sodium nitrite, and carbon monoxide. Both forms cleave the tetrapyrrole molecule exclusively at the alpha meso bridge to form biliverdin IX alpha-isomer. Both forms utilize heme and hemohe as substrates but not intact cytochrome c. HO-1 is also a heat shock protein (Dwyer, *et. al.*, 1989). The rat HO gene contains a functional heat shock element consensus sequence in its promoter region (Mitani, *et. al.*, 1989). It is inducible by a variety of stressful conditions including hyperthermia (Keyse and Tyrell, 1987), ultraviolet radiation, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), sodium arsenite, (Keyse & Tyrell, 1989; Taketani, *et. al.*, 1989), cadmium (Taketani, *et. al.*, 1989), and heme (Yoshida, *et. al.*, 1988; Stocker, 1990). It has been postulated that since heme oxygenase is a sensitive indicator of oxidative stress in some cells (Fligiel, *et. al.*, 1984), it can possibly protect cells by elevating intracellular bilirubin, which is an antioxidant (Stocker, *et. al.*, 1987



**Figure 1. Metabolic Fate Of Heme**

### C. C-6 Glioma Cells As The Model System

Gliomas are prominent nervous system tumors that have received considerable experimental scrutiny in the disciplines of oncology and molecular biology (James, *et. al.*, 1989; Kinzler, *et. al.*, 1987). The C6 Glioma, which is chemically induced from rat brain, has generally been designated as an astrocytoma (Benda, *et. al.*, 1968; Benda, *et. al.*, 1971; & Embree, *et. al.*, 1971). Research has shown that C-6 Glioma cells transdifferentiate or phenotypically change as a function of cell passage. With time, these cells develop more astroglial and less oligodendroglial characteristics, as measured by their decreased expression of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP)(E.C.3.1.4.1) and increased glutamine synthetase (GS)(E.C.6.3.1.2) activity

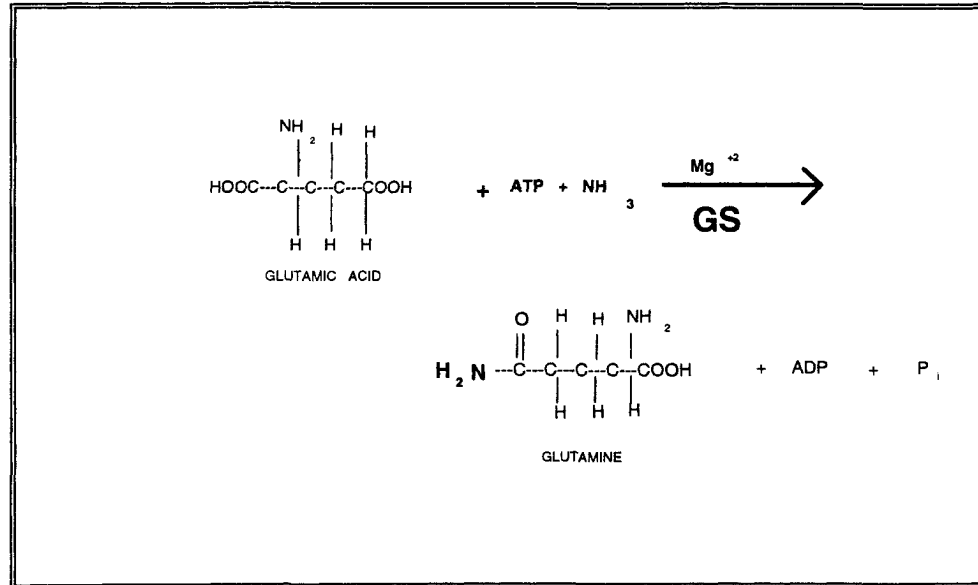
(Parker, *et. al.*, 1981; Parker, 1989). GS is used as a characteristic enzyme marker for astroglial cells (Norenberg and Martinez-Hernandez, 1979) and CNP as an enzyme marker for oligodendrocytes (Poduslo and Norton, 1972).

Rat C-6 glioma cells were chosen for this study to investigate glioma cell properties and function because of the consistency of expression, degree of characterization, and the dependability of culturing conditions of the C-6 cell line (Pfeiffer, *et. al.*, 1977; Vernadakis, *et. al.*, 1986).

## **D. Intracellular Markers**

### **i) Glutamine synthetase**

Studies have shown that the glutamine synthesis (GS) antigen in the adult brain is exclusively localized to astrocytes (Norenberg and Martinez-Hernandez, 1979). These findings were consistent with those demonstrating high glutamine synthetase activity in astrocytes cultures (Schousboe, *et. al.*, 1977). This cytosolic enzyme is distributed approximately equally in both fibrous and protoplasmic astrocytes. A number of experimental workers emphasize the key role of astrocytes in ammonia detoxification and in the metabolism of the putative neurotransmitters  $\gamma$ -amino-butyric acid and glutamic acid (Henn & Hamberger, 1971; Norenberg, 1976; Schrier & Thompson, 1974) (FIGURE 2).



**Figure 2. Glutamine Synthetase Reaction**

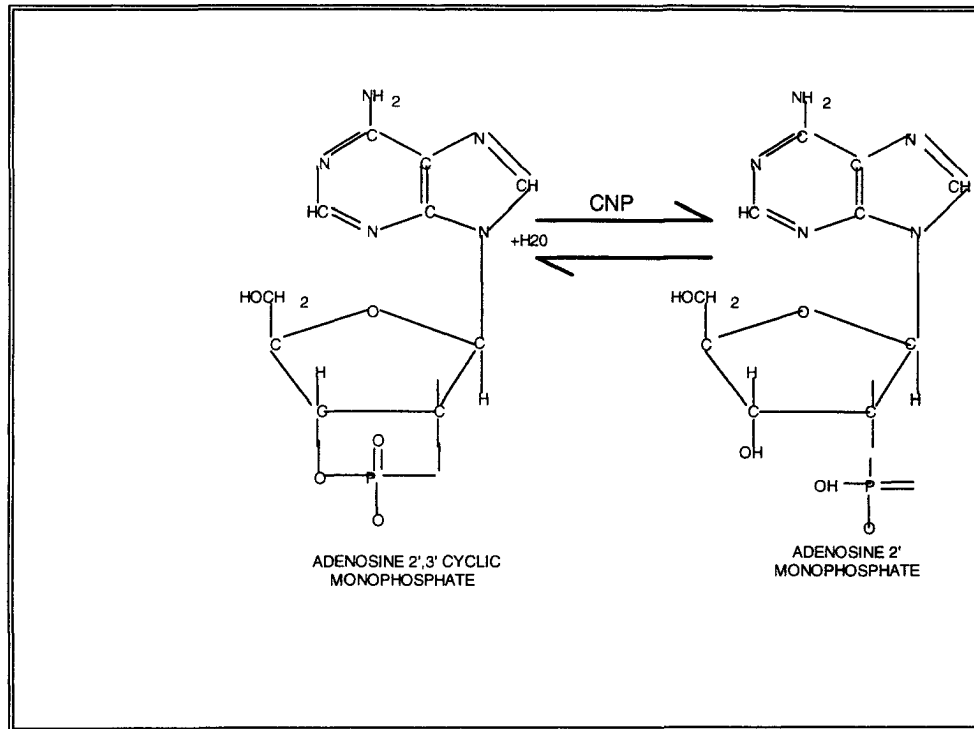
Glutamine synthetase is considered as a reliable astrocyte marker for establishing cellular identification in tissue culture, developmental studies, studies involving neoplastic transformation and differentiation, and as a means of monitoring astrocytes in pathological states (Norenberg and Martinez-Hernandez, 1979).

**ii) 2',3'-Cyclic nucleotide,3'-phosphohydrolase (CNP)  
(EC.3.1.4.1)**

Cyclic nucleotide phosphohydrolase (CNP) is an enzyme or a series of related enzymes capable of hydrolyzing ribonucleoside 2',3'-cyclic phosphates. The enzyme is not capable of hydrolyzing internucleotide bonds nor is it capable of cleaving the more well known 3',5'-cyclic phosphates. (FIGURE 3). The cellular location of the enzyme has been used as an important tool for neurochemists (Zanetta, *et. al.*, 1972). Cyclic nucleotide phosphohydrolase was first shown to be present at high concentrations in myelin rich neural fractions and thus became a marker for myelin. The reason for this



localization became evident later as CNP was found to be localized in high concentrations in the oligodendroglial cell plasma membrane. Therefore, CNP has become an important marker for oligodendroglia (Prohaska, *et. al.*, 1973).



**Figure 3. CNP Reaction**

**e. Specific Objectives**

Whether a correlation exists between heat shock or the stress response and drug resistance in the rat C-6 Glioma cell line has not been reported. We have investigated the heat shock / stress response and drug resistance in the C-6 Glioma cell line with the following objectives:

- (I). To determine whether a drug resistant cell line can be induced with colchicine.
- (II). To characterize differences in the protein content, stress response, and key enzyme levels in the parent (control) and colchicine treated cells.
- (III). To determine whether the drug resistance seen with colchicine pretreatment can be reversed by verapamil.

# MATERIALS AND METHODS

## I. CELL CULTURE:

C6 glioma cells were started from a locally maintained cell line. Cells were recultured approximately every 10 days. Passages used for these studies were taken from passages 70 to 81. Cells were maintained in either Corning (or equivalent) 75 cm<sup>2</sup> flasks or multiple well culture flasks. Usual culture conditions involved growing cells in D-MeM/F-12 medium fortified with 10% fetal bovine serum, penicillin (100 ug/ml), streptomycin (100 ug/ml), and fungizone (250 ug/ml). The medium was changed every 2-3 days, while being maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

## II. REAGENTS AND DRUGS:

Chemicals, reagents, and drugs were obtained from the indicated sources. Rat C-6 Glial tumor cells (American Type Culture Collection Rockville, Md). Acrylamide, N,N,-methylenebis acrylamide (BIS), Trizma base, tetrasodium ethylenediamine tetraacetic acid (EDTA), N,N,N',N'-tetramethylene amine (TEMED), Ammonium persulfate [(NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub>], Sigma 7-9 tris, b-mercaptoethanol (2ME), glycine, glycerol, bromphenol blue, SDS molecular weight markers (14,000-70,000), SDS molecular weight markers (30,000-200,000), Deferroxamine mesylate, Bovine serum albumin, Hemin chloride-bovine, b-Nicotinamide adenine dinucleotide phosphate (b-NADPH), Biliverdin dihydrochloride, Protein, total-micro-Brilliant blue G, Bovine serum albumin standards, Tris-HCL (buffer), Tris maleate (buffer),

monophosphate (2'-AMP), Alkaline phosphatase, Ammonium molybdate, Imidazol hydrochloride (buffer), l-glutamine, hydroxylamine, Adenosine 5' diphosphate (ADP), g-glutamyl-hydroxymate (L-glutamic acid- g-monohydroxymate), Colchicine, Verapamil, (Sigma Chemical Co.; St. Louis, Mo.). Potassium chloride (KCl), Sodium bicarbonate ( $\text{NaHCO}_3$ ), Sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ ) Potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ), Potassium phosphate dibasic ( $\text{K}_2\text{HPO}_4$ ), Sodium hydroxide (NaOH), Hydrochloric acid (HCL), Magnesium chloride ( $\text{MgCl}_2 \cdot 4\text{H}_2\text{O}$ ), Ferric chloride ( $\text{FeCl}_3$ ), Sodium chloride (NaCl), Sulfuric acid ( $\text{H}_2\text{SO}_4$ ), Sodium Arsenate ( $\text{Na}_2\text{HAsO}_4$ ), Sucrose, Manganese chloride ( $\text{MnCl}_2 \cdot 8\text{H}_2\text{O}$ ), Trichloroacetic acid (TCA), Isopropyl alcohol (Isopropanol), Methyl alcohol (methanol), Benzene, Isobutyl alcohol (Isobutanol), Glacial acetic acid, (Fisher Chemical, Fair Lawn, NJ.). Trypan blue stain (0.4%), Earle's balanced salt solution (EBSS), Dulbecco's modified Eagle's medium (D-MEM/F-12), Penicillin-Streptomycin 5000 units/ml, Fungizone (Amphotericin B 250 ug/ml & Sodium deoxycholate 205 ug/ml), Trypsin (2.5%), (Gibco Laboratories, Life Technologies, Inc.). Taxol (Calbiochem, Lajolla, Ca.), Sodium Dodecyl Sulfate (BHD British House, Poole BH15 ITD, England).

### **III. EXPERIMENTAL PROTOCOL:**

#### ***a. Development of a Drug Resistant Cell Line***

Rat C-6 Glioma cell cultures were used throughout this research project. Cells were grown and maintained in Corning (or equivalent) 75 cm<sup>2</sup> flasks and six-well culture plates. The cells were grown in D-MEM/F-12 medium fortified with 10%

fetal bovine serum, Penicillin (100 ug/ml), Streptomycin (100 ug/ml), and Fungizone (250 ug/ml Amphotericin B & 205 ug/ml Sodium deoxycholate). The medium was changed every 2-3 days while being maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

Sets of six 6-well plates were incubated over a 7-10 day period. When microscopic examination had shown that confluent growth had been achieved, the cells were subjected to varying concentrations (250-2000 ng/ml) of colchicine dissolved in D-MEM/F-12 medium for a twenty four hour period. Each 6-well plate contained one well that was used as a solvent control. At the end of the 24 hour treatment period, the D-MEM/F-12 media was aspirated and the cells were harvested by adding 1.0 ml isotonic 0.25% trypsin. The trypsinized cells were then pipetted into separate prelabeled tubes containing 2-3 volumes of ice cold D-MEM/F-12 medium. The response of the cells to colchicine was then assessed by microscopically counting the cells (as described in Appendix B-Trypan Blue Cell Counting Procedure). The EC<sub>50</sub> of colchicine (the dose in which there is a 50% inhibition of growth of the cells) was determined by using the Pcnolin software program. Correlation coefficient (R-values) were 0.9 or better for EC<sub>50</sub> determination.

***b. Assessment of Drug Resistance***

Six-well culture plates were exposed to the EC<sub>50</sub> concentration of colchicine for 24 hours. Drug exposure was then terminated and the cells were grown to confluence in the standard D-MEM/F-12 medium. Drug resistance was assessed by subjecting the cells to varying concentrations (100-5000 ng/ml) of taxol. Cells were

harvested and counted as described previously. Taxol EC<sub>50</sub> values were determined for both the colchicine treated cells and the control (PNT) cells.

***c. Induction of Stress***

Confluent cultures of the colchicine treated and parent (control) cells were subjected to heat stress by incubating them at 42° C for 30 minutes. 24 hours later the cultures were harvested and counted. Cells were then prepared for protein and enzyme determinations (see part 4).

***d. Assays Procedures***

The details of the analytical procedures are described in Appendix B.

***i) Cell Counts:***

Two hundred microliters (200 ul) of the previously trypsinized cell suspension was pipetted into a tube containing 300 ul of 0.85% saline and 500 ul Trypan blue. A sufficient volume of the Trypan blue cell suspension was transferred to a hemacytometer. All of the cells in each of the four corner and center grids were counted for each sample. The average number of cells obtained was multiplied by 5 to correct for the dilution factor. To obtain the number of cells per milliliter in the original solution, the corrected number was multiplied by 10<sup>4</sup> (Bauer H, et.al., 1975).

***ii) Protein Analysis:***

A microprotein technique (Sigma cat.#610-A) that employs Brilliant Blue G was used (Bradford, 1976). The Brilliant Blue G dye (Coomassie Blue) reacts almost immediately with protein to form a blue colored protein dye complex. The amount of color produced is proportional to the protein concentration. In the assay procedure, the C-6 Glioma cell homogenates along with protein standards were first solubilized by heating at 80°C for 10 minutes in 0.05N NaOH. After neutralization with 1.21N

HCL, the blanks, standards, and samples were reacted with protein dye solution. The absorbance (O.D.) of each was measured at 595 nm. The amount of protein in each sample was calculated from the slope of the regressed line in which the absorbance (O.D.) was plotted against the concentration (mg/ml) of the protein standards.

iii) Heme Oxygenase Activity

The enzymatic procedure used was the spectrophotometric method to measure the formation of bilirubin (Tenhunen *et. al.*, 1968) as modified by Lincoln *et.al.* (1988). The method relies on the use of the endoplasmic reticular fraction of the C-6 Glioma cell homogenate and the addition of previously isolated rat liver cytosolic fraction as a source of biliverdin reductase (see Heme Oxygenase Assay-Appendix B). Hemin was then added to the reaction mixture. The presence of heme oxygenase in the C-6 Glioma cell homogenates, oxidatively catalyzes the conversion of hemin to bilirubin. Subsequently, the presence of biliverdin reductase and  $\beta$ -Nicotinamide adenine dinucleotide phosphate ( $\beta$ -NADPH) in the reaction mixture reduced biliverdin to bilirubin. The conversion of hemin via the two step process to bilirubin was measured spectrophotometrically at 453nm. The heme oxygenase activity (umol/min) was determined from a standard curve prepared with bilirubin standards. The heme oxygenase activity was expressed as pmol/min/mg of protein.

iv) Glutamine Synthetase Activity (GS):

GS was assayed by the colorimetric method of Meister (1985). In this procedure, the enzymatic activity in the C-6 Glioma cell homogenates and prepared standards were followed by the use of the  $\gamma$ -glutamyl transfer reaction in which glutamine synthetase catalyzes the conversion of L-glutamine in the presence of

hydroxylamine, catalytic quantities of Adenosine 5' diphosphate, and inorganic arsenic to  $\gamma$ -glutamylhydroxymate; which gives a characteristic color reaction upon addition of ferric chloride. The absorbance (O.D.) for each of the standards and samples was measured spectrophotometrically at 555nm. The  $\gamma$ -glutamylhydroxymate activity (umol/min) was determined from a standard curve prepared with  $\gamma$ -glutamylhydroxymate. The  $\gamma$ -glutamylhydroxymate activity was expressed as nmol/min/mg protein.

v) 2'3'-Cyclic Nucleotide 3'Phosphohydrolase (CNP) Activity:

The procedure used was the spectrophotometric method to measure the formation of 2'-adenosine monophosphate (Prohaska *et. al.*, 1973). Aliquots of C-6 Glioma cell homogenates and standards were incubated with 2',3'-cyclic adenosine monophosphate to yield 2'-adenosine monophosphate. Alkaline phosphatase was added to cleave inorganic phosphate from the 2'-adenosine monophosphate. The inorganic phosphate was reacted with ammonium molybdate and then the complex was extracted in isobutanol/benzene (1:1). The yellow color produced in the organic layer was then measured spectrophotometrically at 410nm. The 2',3'-cyclic nucleotide phosphohydrolase activity (umol/min) was determined from a standard curve prepared with 2'-adenosine monophosphate standards. The CNP activity was expressed as umol/min/mg of protein.

vi) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)



The SDS-PAGE procedure used utilized the tris-glycine discontinuous system (Laemmli, 1970) as modified by Dr. Ralph Judd (1987,1988). C-6 Glioma cell homogenates, including the parent strain, the colchicine treated strain, and cultures exposed to heat shock, were subjected to SDS-PAGE ( as described in Appendix B) along with commercially prepared molecular weight protein markers. After fixing in 50% methanol: 7% acetic acid: 43% distilled water, the proteins were visualized with Coomassie Brilliant Blue R. The technique was used for this study to visually assess the presence of heat shock proteins including Hsp 32 (heme oxygenase), Hsp 70, and Hsp 90.

**e. The Effect Of Verapamil On Drug Resistance**

Four twenty-four well culture plates containing the colchicine treated strain were treated with varying concentrations (1 uM, 5 uM, and 10 uM in D-MEM/F12 medium) of verapamil for 24 hours. At the end of 24 hours, the colchicine treated cells (including appropriate controls) was subjected to varying concentrations (100-2000 ng/ml) of taxol for 24 hours. Finally, the cells were trypsinized, harvested and then placed in Trypan blue stain. Cells were then counted microscopically to determine the effects of taxol on cell growth.

**f. Statistics**

Student t-tests were used to determine significant differences between the parent (control) cells and the colchicine treated cells. ANOVA and Scheffe's tests were used to determine significant differences among multiple treatment means (both stressed and unstressed).  $p < .05$  = significant difference. PCnonlin was used to determine  $EC_{50}$  values for taxol.

## RESULTS

**OBJECTIVE I:** *To determine whether a drug resistant cell line can be induced with colchicine.*

Throughout this study, the Trypan Blue exclusion staining technique was used to determine cell viability. While colchicine decreased cell counts, no indication of cell death nor cellular debris was detected.

Figure 4 shows the effect of varying concentrations of colchicine on cell counts. Cell counts decreased from  $87.3 \pm 3.3$  % of control at a concentration of 250 ng/ml to  $33.6 \pm 3.0$  % of control at a concentration of 2000 ng/ml. A PCNonlin statistical program was used to calculate the  $EC_{50}$  of colchicine (480 ng/ml).

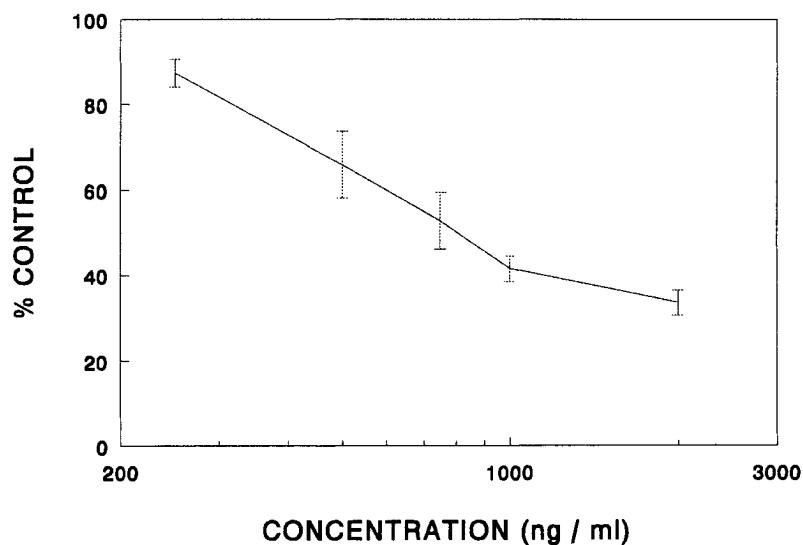


Figure 4. Effects of colchicine on cell counts. All values are mean  $\pm$  s.e.m. Each data point represents  $n=6$ . Cell counts decrease from  $87.3 \pm 3.0\%$  of control at 250 ng/ml to  $33.6 \pm 3.0\%$  of control at a concentration of 2000 ng/ml.

An additional effect of colchicine was noted in a small study ( $N = 2$ ), comparing the total number of cells counted at 24 hour intervals in the control cells (PNT) and the colchicine treated cells (CLT). Results shown in Figure 5 indicate that cell counts, after exposure to colchicine for 24 hours, decreased for approximately 72 hours. At ninety six (96) hours, cell counts in the colchicine treated cells is approximately equal to that of the control cells (PNT).

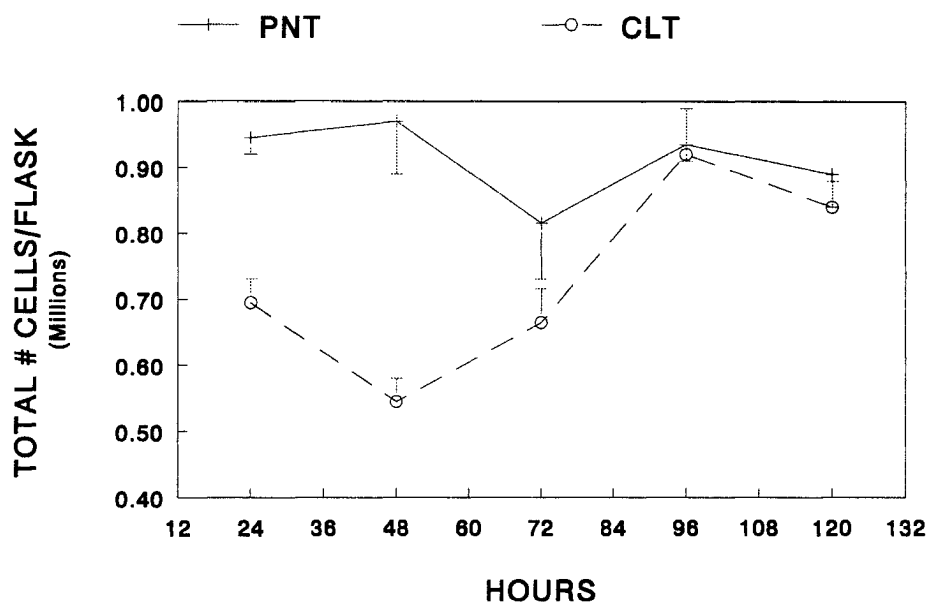


Figure 5. Effect of colchicine pretreatment on cell counts. PNT = control cells; CLT = colchicine treated cells. Each point represents the mean value.  $n = 2$ . At 96 hours both the control and colchicine treated cell counts were at approximately  $9.0 \times 10^5$  cells per flask.

Taxol was much less effective in cells previously exposed to colchicine (Fig.6). The control cells shows less resistance to taxol (decrease in the mean cell counts as a % of control) than the colchicine treated cells. The taxol concentration

ranged from 0-2000 ng/ml in the control cells and ranged from 0-5000 ng/ml in the colchicine treated cells. Using a PCNonlin statistical program, the taxol  $EC_{50}$  in the parent (control) cells was determined to be  $82.0 \pm 23.5$  ng/ml. The taxol  $EC_{50}$  in the colchicine treated cells was determined to be  $1490.3 \pm 157.7$  ng/ml. Values are expressed as mean  $\pm$  95% confidence interval.

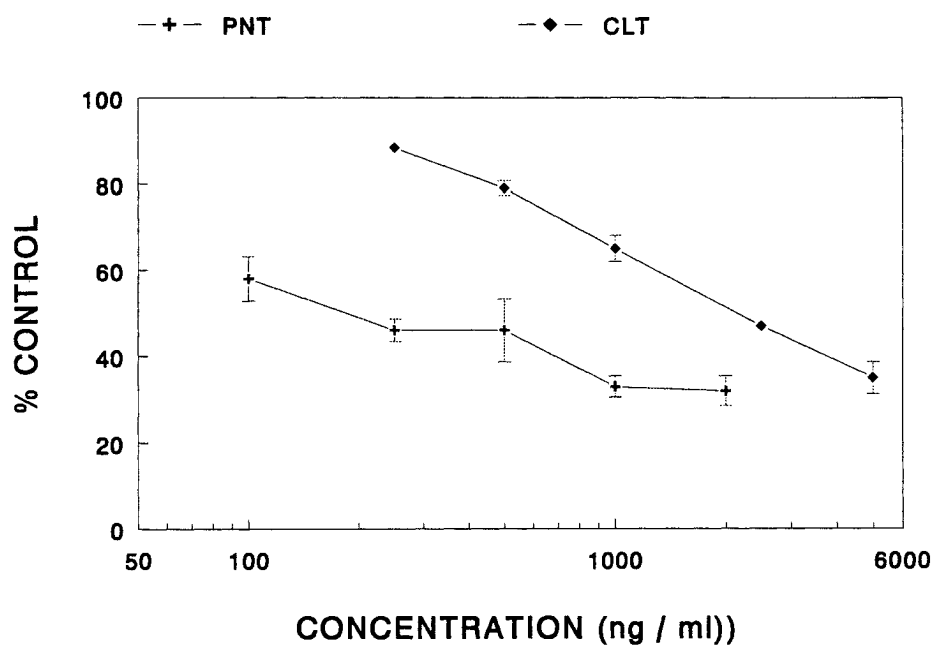


Figure 6. Effect of taxol on cell counts. PNT = control cells ( $EC_{50} = 82 \pm 23.5$  ng/ml). CLT = colchicine treated ( $EC_{50} = 1490.3 \pm 157.7$  ng/ml). % control values = mean  $\pm$  s.e.m.  $EC_{50}$  values = mean  $\pm$  95% confidence interval.

***Objective II: To characterize differences in the protein content, stress response, and key enzyme levels in the parent (control) and colchicine treated cells.***

**a. Heat shock proteins:**

Cell cultures from both treatments, stressed (heat shocked) and unstressed, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Visual examination of Coomassie stained gels (pages 34-37) showed the presence of several bands with similar molecular weights to the heat shock proteins-- Hsp 28, Hsp 60, Hsp 70, Hsp 90. A band having a molecular weight of approximately 32 kDa (Hsp 32 -heme oxygenase) was apparent on all gels.

However, a perceptible increase in the well known heat shock protein Hsp 70 was not observed on either the stressed or the unstressed cultures. Densitometric scans were performed which compared the stressed colchicine treated cells (CLT + HS) to a known, commercially prepared, protein standard molecular weight marker mixture. The densitometric scan on the gel from the colchicine treated cells showed the presence of several peaks having similar retention times to the peaks seen on the scan of the molecular weight marker mixture. However, induction of stress (increase in Hsp 70 or Hsp 32) was not observed on the scan from the colchicine treated cells. (pages 38-39).

**b. Protein content:**

Measurement of the amount of protein per cell indicated a significantly higher concentration in the colchicine treated cells ( $3.73 \pm .15 \times 10^{-7}$  ug per cell). The amount of protein in the control cells was  $3.2 \pm .20 \times 10^{-7}$  ug per cell (Fig. 7).

**c. Heme oxygenase (HO):**

Colchicine pretreatment increased HO activity ( $58 \pm 3$  pmol/min/mg protein) (Fig 8). Heme oxygenase activity in the unstressed control cells was determined to be ( $33 \pm 7$  pmol/min/mg protein). Heat shock treatment significantly increased heme oxygenase activity in the control cells ( $60 \pm 6$  pmol/min/mg protein). HO activity appeared to increase when the CLT cells was heat shocked ( $53 \pm 7$  pmol/min/mg protein), however no significant difference was detectable ( $p = .059$ ).

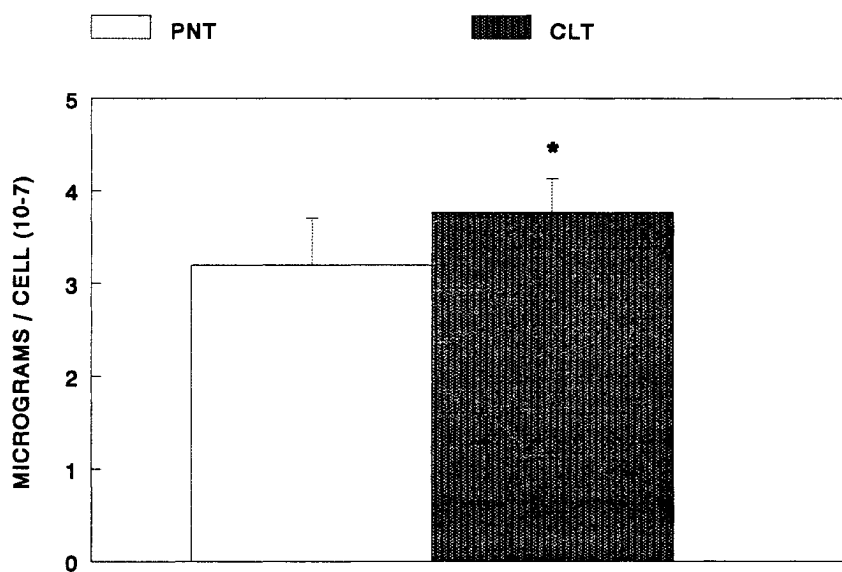


Figure 7. Effect of colchicine on cellular protein content (ug protein x  $10^{-7}$ ) per cell. Cells harvested 24 hours after colchicine removed from medium. Values are mean  $\pm$  s.e.m. \* = significant difference ( $p < 0.05$ ). PNT and CLT are defined in Fig. 5.

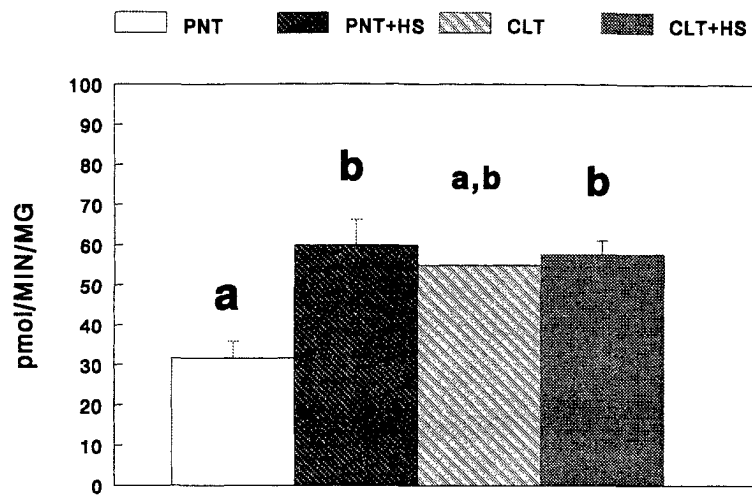


Figure 8. Effect of colchicine and heat shock on heme oxygenase activity. PNT and CLT are defined in Fig. 5. HS = heat shock. Bars not sharing a common letter are significantly different ( $p < 0.05$ ). All values are mean  $\pm$  s.e.m.  $n = 6$ .

**d. Glutamine synthetase (GS):**

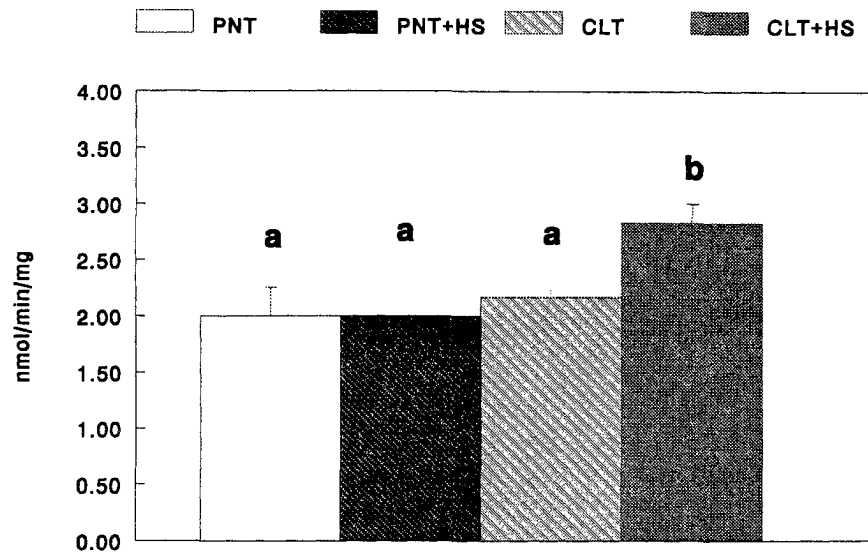


Figure 9. Effect of colchicine and heat shock on glutamine synthetase activity. No significant difference in GS activity was noted between the PNT, PNT + HS, and the CLT treated cells. PNT, CLT and HS are defined in Figs. 5 & 8. See Fig. 8 for explanation of statistics.

Glutamine synthetase in the unstressed control cells (PNT) was determined to be  $2.0 \pm 0.2$  nmol/min/mg protein. GS activity in the stressed control cells (PNT + HS) was 2.0 nmol/min/mg protein. In the unstressed colchicine treated cells (CLT) no significant increase in GS activity was noted ( $2.17 \pm 0.17$  nmol/min/mg protein). A significant increase ( $p < .05$ ) in GS activity was determined in the stressed colchicine treated cells (CLT + HS) ( $2.83 \pm 0.17$  nmol/min/mg protein). No statistical



significance was noted between the control cells, the stressed control cells, or the colchicine treated cells (Fig. 9).

**e. Cyclic Nucleotide Phosphohydrolase (CNP):**

Colchicine did not significantly increase CNP activity (Fig. 10). No significant difference was noted between the unstressed control cells (PNT) and the unstressed colchicine treated cells (CLT)( $0.218 \pm 0.015$  umol/min/mg protein). The CNP activity in the stressed colchicine cells were significantly higher than either the stressed or unstressed control cells. Heat stress did not significantly increase the CNP activity in the colchicine treated cells ( $0.249 \pm 0.006$  umol/min/mg protein vs.  $0.218 \pm 0.015$  umol/min/mg protein in CLT), although the former value was significantly higher than values obtained with the control cells.

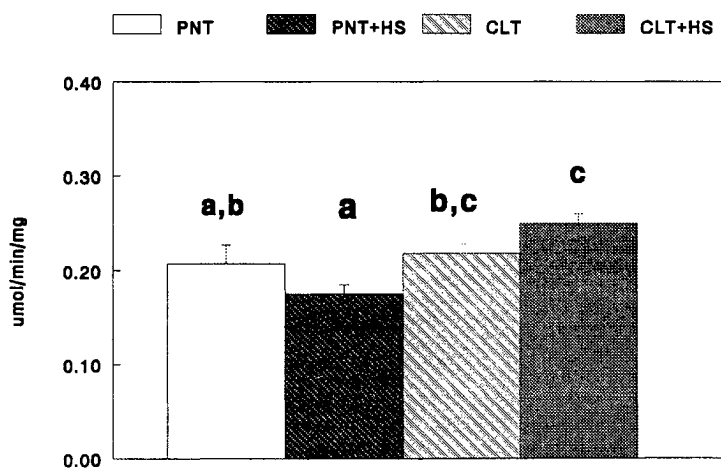


Figure 10. Effect of colchicine and heat shock on CNP activity. Only the stressed colchicine treated cells showed increased CNP activity. See Fig. 8 for details of legends and statistics.

**Objective III: To determine whether the drug resistance seen in the colchicine treated strain can be reversed by verapamil.**

The colchicine treated cells were pretreated with varying concentrations of verapamil (1  $\mu$ M, 5  $\mu$ M, & 10  $\mu$ M). A significant increase in cell counts was seen in the colchicine treated cells in the presence of 10  $\mu$ M of verapamil (Fig. 11).

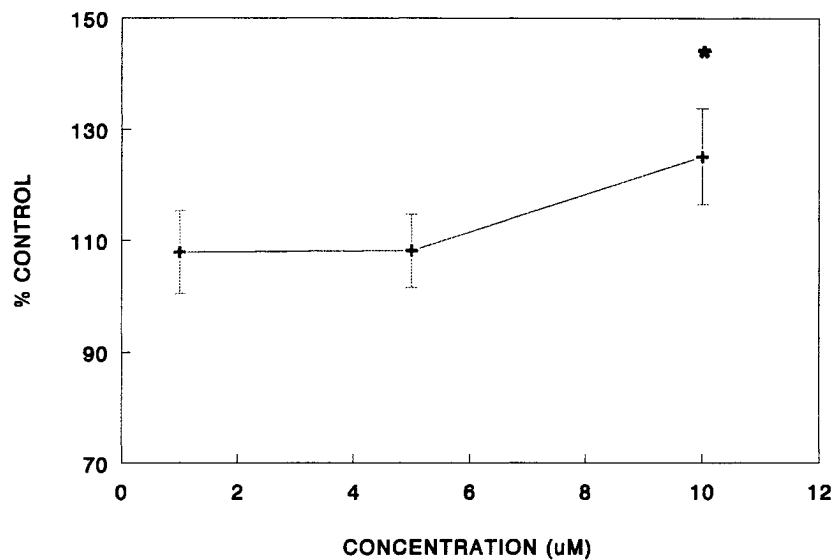


Figure 11. Effect of verapamil on cell counts in the CLT cells. Cells exposed to 1, 5, or 10  $\mu$ M verapamil for 24 hours prior to counting. \* = significantly different from other concentrations ( $p < 0.05$ ). Values are mean  $\pm$  s.e.m.  $n = 6$ .

Verapamil, at a 10  $\mu$ M concentration, was effective in decreasing colchicine resistance to taxol (Fig. 12). Figure 13 compares the effects of verapamil on taxol  $EC_{50}$  values in the colchicine treated cells to the original  $EC_{50}$  data shown in Figure 6. The taxol  $EC_{50}$  of passage 81 was  $9103 \pm 2088$  ng/ml; at 1  $\mu$ M verapamil pretreatment the

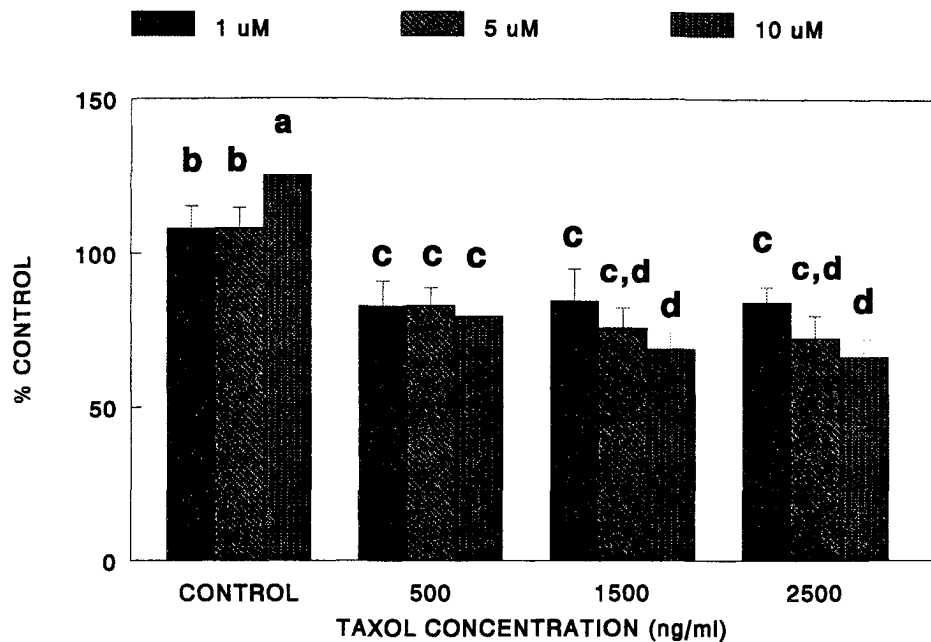


Figure 12. Effect of verapamil (1, 5, and 10  $\mu\text{M}$ ) on the CLT strain's response to taxol. 10  $\mu\text{M}$  verapamil significantly decreases the colchicine resistance to taxol. See Fig. 8 for details of statistics.

taxol  $\text{EC}_{50}$  was  $676 \pm 240$  ng/ml; at 5  $\mu\text{M}$  verapamil pretreatment the taxol  $\text{EC}_{50}$  was  $439 \pm 56$  ng/ml; and at 10  $\mu\text{M}$  verapamil pretreatment the taxol  $\text{EC}_{50}$  was  $72 \pm 50$  ng/ml. By comparison, the taxol  $\text{EC}_{50}$  in the control cells was  $82 \pm 23$  ng/ml; and the taxol  $\text{EC}_{50}$  in the colchicine treated cells was  $1490 \pm 158$  ng/ml. Verapamil produced a concentration-dependent reversal in the colchicine-induced drug resistance to taxol. This reversal was essentially complete in presence of 10  $\mu\text{M}$  verapamil.

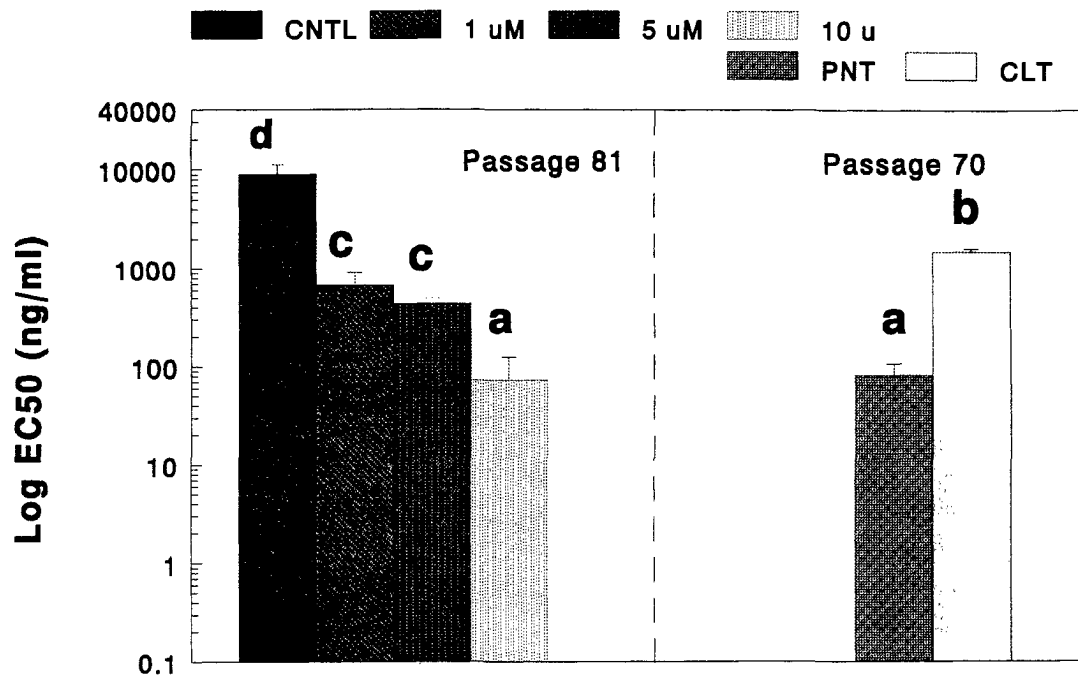


Figure 13. Effects of verapamil on the response of CLT to taxol.  $EC_{50}$ 's for Passage 81 were determined following exposure to the indicated concentration of verapamil. CNTL = CLT cells not exposed to verapamil. CLT and PNT are defined in Fig. 5. Bars represent mean  $\pm$  95 % confidence interval. Values for Passage 70, both PNT and CLT, are shown on the right side of the graph.

PARENT STRAIN + HEAT SHOCK

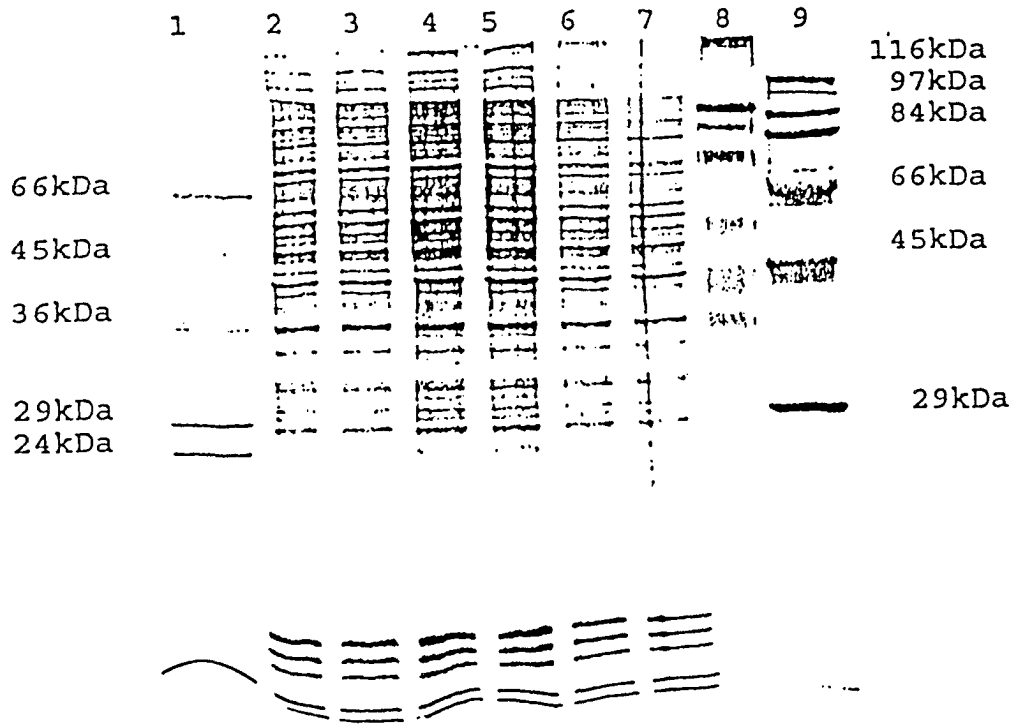


Fig. 14. Separation of proteins after heat shock (42°C for 30 minutes) by SDS-Page. Lane 1, SDS-Page Molecular Weight marker standard I; lanes 2-7, parent strains I-VI; lane 8, SDS- Page molecular weight marker standard II; lane 9, SDS-Page molecular weight marker standard III.

PARENT STRAIN

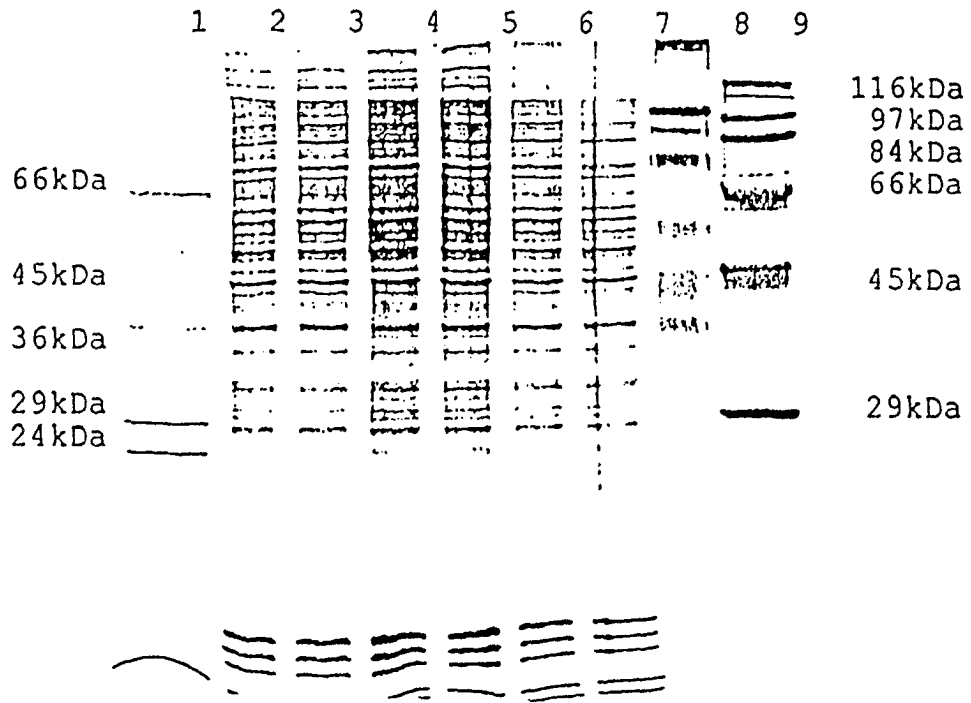


Fig. 15. Terms are the same as in figure 14.

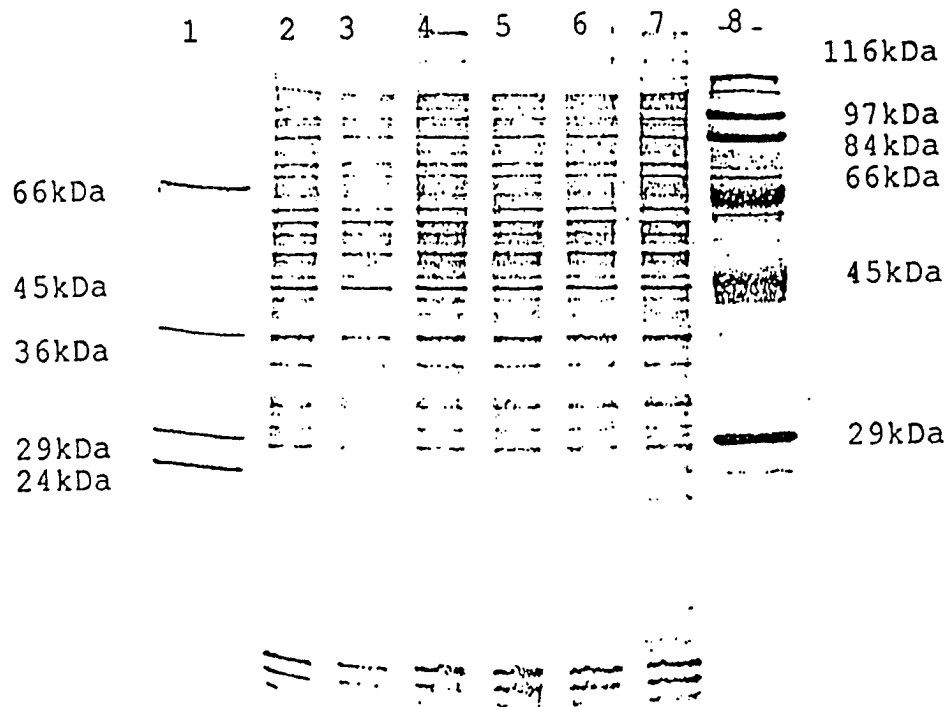


Fig. 16. Terms are the same as in figures 14 and 15, except that SDS-PAGE molecular weight marker standard II was not run.

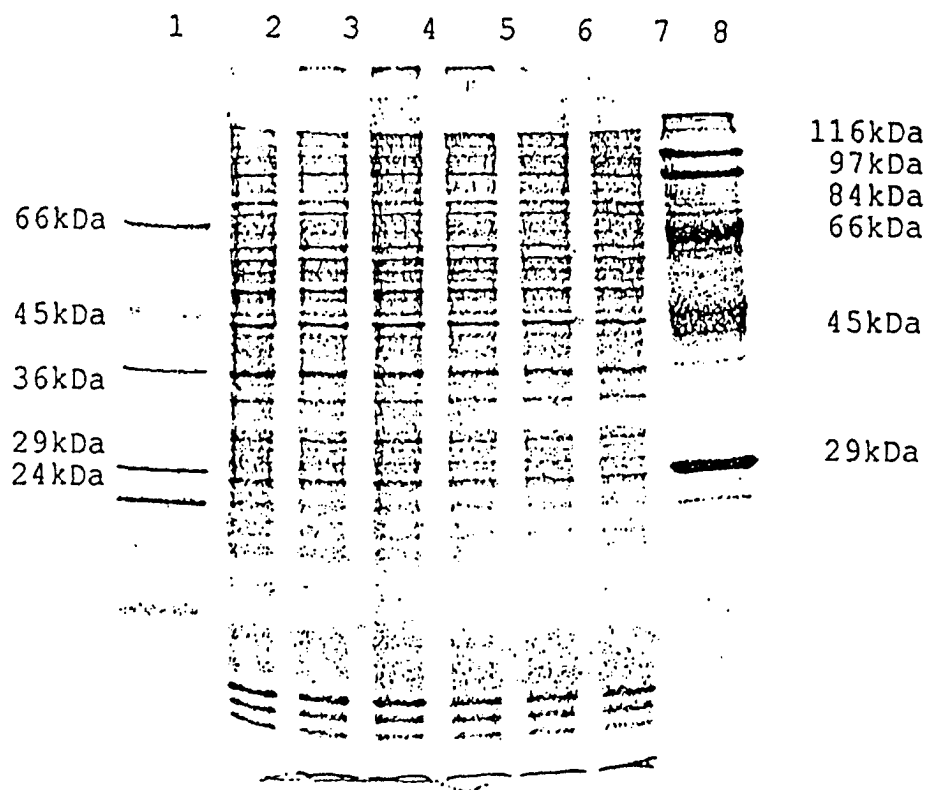
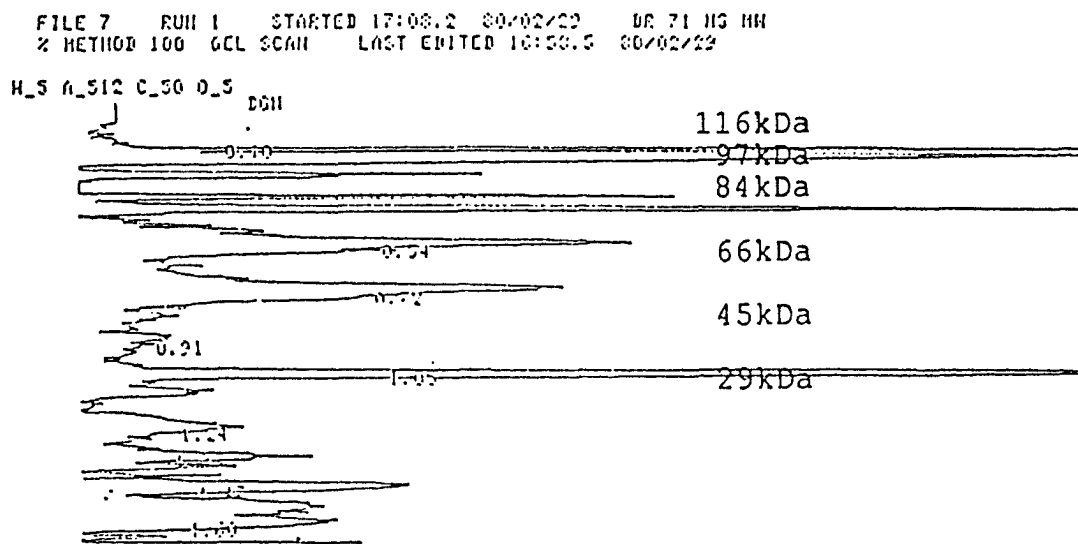


Fig. 17. Terms are the same as in figure 16. Heat shock conditions are the same as in figure 14.



Densitometric Scan  
SDS-PAGE Molecular Weight Marker III



FILE 7 RUN 1 STARTED 17:08.2 00/02/29 DR 71 HS MH  
% METHOD 100 GEL SCAN LAST EDITED 16:58.5 00/02/29

RT	AREA	HEIGHT	DC	AREA PERCENT	HEIGHT PERCENT
0.10	2465670	10761.3975	U	35.1468	38.6462
0.54	10224571	2689.9987	T	14.4581	2.6381
0.72	12464450	2315.6436	T	17.6254	8.3589
0.91	1788580	416.4582	T	2.5291	1.5038
1.05	11504969	7832.5312	U	16.2686	28.2736
1.24	2975936	758.1867	T	4.2081	2.7267
1.36	3499312	1032.4911	U	4.9482	3.7271
1.47	3387848	1477.9208	U	4.7906	5.3350
1.60	16379	433.0370	U	0.0232	1.5812

Exit Report Reprint Print Del All Delete List

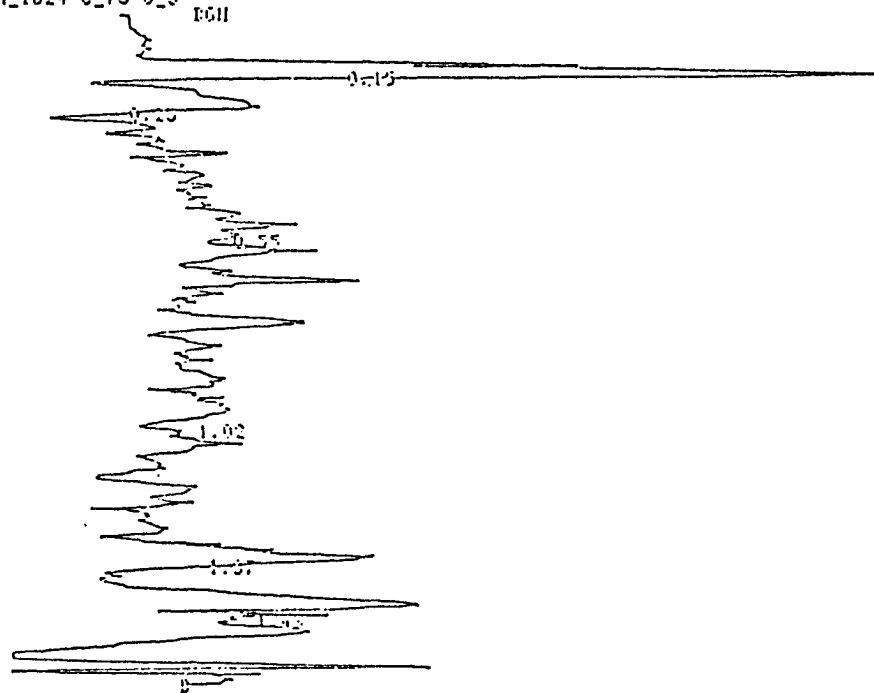
2 PEAKS > AREA REJECT 70718816 TOTAL AREA  
2 PEAKS > HEIGHT REJECT 2.770260e+04 TOTAL HEIGHT

Fig. 18. Conditions of scan are as noted above. Concentration of proteins in the SDS-PAGE molecular marker standard were at approximately 1 microgram per microliter.

Densitometric Scan  
Drug Resistant Strain + Heat Shock

FILE 10 RUN 1 STARTED 17:13.0 00/02/29 DR 71 HS LANE 1  
% METHOD 100 GEL SCAN LAST EDITED 17:13.0 00/02/29

W\_5 A\_1024 C\_75 0\_5



FILE 10 RUN 1 STARTED 17:13.0 00/02/29 DR 71 HS LANE 1  
% METHOD 100 GEL SCAN LAST EDITED 17:13.0 00/02/29

RT	AREA	HEIGHT	PC	AREA PERCENT	HEIGHT PERCENT
0.15	15659376	7710.1733	T	14.2976	40.0232
0.25	6262658	1246.5654	U	5.7160	10.3000
0.55	44889456	2269.0437	T	40.9857	11.6889
1.02	21527520	1441.9749	T	19.6354	7.6300
1.37	11173468	2645.6165	U	10.2018	13.9990
1.48	10012270	2939.2776		9.1416	15.5528

C PEAKS > AREA REJECT 103324744 TOTAL AREA  
C PEAKS > HEIGHT REJECT 1.3398658404 TOTAL HEIGHT

Fig. 19. Conditions of Gel scan are noted above.

## DISCUSSION

***Objective I: To determine whether drug resistant cell line can be induced with colchicine.***

The data shown in Figure 4 was used to determine the concentration of colchicine to be used for further studies on drug resistance. The calculated colchicine  $EC_{50}$  value of 480 ng/ml was selected to induce drug resistance in this C-6 glioma model. The concentration of colchicine chosen initially inhibited cell growth during the 24 hour exposure. Seventy-two (72) hours after removal of the colchicine, the C-6 glioma cell growth had returned to a level ( $9.0 \times 10^5$  cells per  $75 \text{ cm}^2$  flask) which was not significantly different from the control ( $9.14 \times 10^5$  cells per  $75 \text{ cm}^2$  flask) (Fig. 5). In addition, the  $EC_{50}$  concentration did not appear to be lethal to the glioma cells.

Podda, *et. al.* (1992) reported that colchicine kills MDR-negative cells. However, they also did not see cell death in cell cultures which were continuously exposed to colchicine for 3 days. Kane and Gottesman, (1993), reported that in colonies continuously exposed to colchicine for 7-14 days, drug sensitive cells were not killed by colchicine but were prevented from dividing.

Previous studies have shown colchicine to be a MDR inducible drug (Podda, *et. al.*, 1992). In the presence of colchicine, cells that are not multiple drug resistant become multinucleated and cell division is inhibited. Cells that express MDR become resistant to the drug. By increasing the selective pressure (colchicine concentration) on those cells, they express progressively more MDR gene product to

1988) or by enrichment for cells in a population that already express high levels of MDR (Kane, *et. al.*, 1989).

Evidence that colchicine treatment induced drug resistance in C-6 glioma is strongly indicated by the shift of the taxol concentration response curves (Fig. 6) and the 15-fold increase in  $EC_{50}$ . Our study is apparently the only study known that has established taxol  $EC_{50}$  values in C-6 glioma. This level of resistance is higher than that seen by other investigators in different cell lines. Kelland, *et. al.*, (1992) compared the cytotoxic properties of taxol in nine human ovarian carcinoma cell lines. They reported that exposure of the ovarian carcinoma cell lines to taxol for two (2) hours showed a 3-9 fold increase in taxol  $EC_{50}$ ; whereas, a continuous 96-hour exposure revealed a 2-6 fold increase. In comparing our study to Kelland's, it is apparent that the taxol  $EC_{50}$  concentrations may vary significantly from cell line to cell line. Differences may best be explained by the studies of Pastan, *et. al.*, (1991) in which several fold differences may be seen based on the degree of expression of p-glycoprotein. Tumors derived from colon, kidney, liver, and pancreas usually have high levels of p-glycoprotein (MDR1), a reflection of the high level in the normal cells from which the tumor arose. Expression of the MDR1 gene is sometimes high in leukemias, lymphomas, and some other cancers derived from tissues that do not normally express the gene. Increased MDR1 expression has been observed in drug-resistant leukemias, myelomas, ovarian cancer, breast cancers, sarcomas, and neuroblastomas (Goldstein, *et. al.*, 1989), and has often been predictive of unresponsiveness to treatment.

Our results indicate that we have developed drug resistant C-6 glioma cells. Noting that the control and CLT cells differ in their response to taxol, our next step then was to characterize changes in certain key intracellular components.

***Objective II: To characterize differences in the protein content, stress response, and key enzyme levels in the control and colchicine treated cells.***

**a. Heat Shock Proteins:**

Our results, using SDS-PAGE, showed the presence of several electrophoretic bands, on Coomassie stained gels, having similar molecular weights to the heat shock proteins—Hsp 28, Hsp 60, Hsp 70, and Hsp 90. Commercially prepared protein standard molecular weight marker mixtures were analyzed in conjunction with the stressed and unstressed parent and colchicine treated strains. A band having a molecular weight of approximately 32 kDa (possibly Hsp 32--heme oxygenase) was apparent on all gels. Visual examination of the gels was insufficient in determining clear differences in protein electrophoretic patterns between the control cells and the colchicine treated cells. A densitometric scan on the gel from the stressed colchicine treated cells showed the presence of several peaks having similar retention times to the peaks seen on the scan of the molecular weight marker mixture. The induction of stress, as characterized by increases in peak areas at retention times similar to compounds having molecular weights at 32 kDa or 70 kDa, was not observed (pages 38-39).

At the present time, two isoforms of heme oxygenase exist; HO-1 and HO-2 -- both forms having nearly identical molecular weights. HO-1 apparently is the only

inducible form (Maines, *et. al.*, 1985). Taketani, *et. al.*, (1989) were not able to distinguish the isoforms of heme oxygenase in rat liver fractions using gel electrophoresis without [<sup>35</sup>S]methionine labeling of the proteins and immunoblotting. Trakshel and Maines (1989) used antibodies to characterize differences in rabbit HO-1 and HO-2. Neither, the [<sup>35</sup>S] Methionine labeling of proteins nor antibodies to HO-1 or HO-2 techniques were available in our lab at the time this study was carried out.

**b. Protein content**

One of the criteria used in this study to characterize differences between the control and colchicine treated cells was the amount of protein per cell. Our study showed a significant increase in the amount of protein per cell in the colchicine treated cells ( $3.73 \pm 0.15 \times 10^{-7}$  ug per cell) versus the amount of protein found in the control cells (figure 7). The amount of protein per cell in our control cells (PNT) ( $3.20 \pm 0.15 \times 10^{-7}$  ug per cell) was almost double the level reported by Parker, *et. al.*, (1980) in a 2-B clone C-6 glioma cell line ( $1.86 \pm 0.13 \times 10^{-7}$  ug per cell). The difference in the two protein levels may be due to the fact that our glioma cell line was from the American Culture Collection. This line may express a higher level of protein than the 2-B clone.

**c. Heme Oxygenase (HO):**

Dwyer, *et. al.*, (1992) characterized heme oxygenase as a stress protein that is inducible by heat shock (stress). In our cell cultures (passage #70) harvested 24 hours after heat stress, a heat shock response was clearly demonstrated by the increase in heme oxygenase activity. Basal HO activity in the untreated control cells (PNT) was  $33 \pm 7$  pmol/min/mg protein (Fig. 8). Heat shock (stress) increased HO activity

approximately 2-fold in both the control (PNT + HS) and the colchicine treated cells (CLT + HS). In addition, the unstressed colchicine treated cells (CLT) showed approximated a 1.5 fold increase in HO activity, indicating that colchicine also induced a stress response.

Dwyer, *et. al.*, reported a 1.9 fold increase in HO activity in rat forebrain astrocytes after a 20 min. heat stress. They also noted a 7 fold increase in HO activity after 2 hours of heat stress. In both instances cells were harvested four (4) hours after a heat stress of 42°C. Ewing, *et. al.*, (1992) reported a 20 fold increase in HO activity in rat brain after a 20 minute heat stress at 42°C. Cells in their study were harvested six (6) hours post treatment. Our data indicates that a typical heat stress response has occurred, i.e., approximately a doubling in HO activity as a result of heat stress. In our laboratory, cells were harvested 24 hours after a heat stress of 42°C for 30 minutes. It is apparent that the extent of the activity may be related to the severity of the insult (length of time of the stress 15 min, 30 min, 2 hour, etc.) or the types of tumor cells analyzed (Steels, *et. al.*, 1992).

Our results also indicated that colchicine causes an increase in HO activity in C-6 glioma. This increase seems to parallel drug resistance; at least there appears to be some correlation between drug resistance and increased HO activity in cell cultures at passage #70. This correlation may or may not exist for cell passage #81 since enzyme activity was not determined in these cells. Our data does show, however, that cell passage #81 was even more resistant than the colchicine treated cells at cell passage #70. The correlation between drug resistance and increased HO activity seen

at cell passage #70 may be a result of the induction of the multiple drug resistance gene (MDR1) in response to heat shock. Kioka, *et. al.* (1992) has reported that heat shock does increase MDR1 levels in a drug-resistant mouse tumor cell line.

**d. Glutamine Synthetase Activity (GS):**

Glutamine synthetase is considered an enzyme marker for astroglia. This C-6 glioma strain often appears to change more towards astroglial character as it ages (Parker, *et. al.*, 1980). At cell passage #21, Vernadakis, *et. al.* (1986) noted GS activity of approximately 1.25 nmol/min/mg of protein for C-6 glioma cells grown 10-14 days in culture. At cell passage #70, used in our study, the GS activity of the cells was  $2.0 \pm 0.2$  nmol/min/mg protein (Fig. 9). Vernadakis, *et. al.*, (1986) reported a GS activity at cell passage #82 of approximately 3.7 nmol/min/mg protein in C-6 glioma. The GS activity seen in the unstressed control cells (PNT) at cell passage #70 might be expected as it is intermediate between the older and younger cultures reported by the Vernadakis group.

The results of our study indicate that GS does not respond to heat stress or colchicine in the same manner as heme oxygenase. The GS activity in our cell passage #70 does not appear to have paralleled drug resistance. There are no reports in the literature to indicate that GS is a heat shock protein inducible by stress. Studies have shown that various compounds, i.e., glucocorticoids (Pishah and Phillips, 1980), sodium butyrate (Weingarten, *et. al.*, 1981),  $\beta$ -adrenergic agonists and dibutyryl cyclic AMP (Browning and Nicklas, 1982) induce GS activity in C-6 glioma. It may be that colchicine by itself slightly induces GS activity, while heat stress provides some uncharacterized additive factor. There was no increase in GS activity in the



parent strain subsequent to heat stress. Our results did show a significant increase in GS activity in the heat-shocked colchicine treated cells (CLT + HS), however, no increase in GS activity was seen in the control cells, and colchicine did not significantly increase enzyme activity in the absence of heat shock (Fig. 9).

**e. Cyclic Nucleotide Phosphohydrolase Activity (CNP):**

Colchicine treatment did not appear to change CNP activity (Fig. 10). The activity in the stressed colchicine treated cells is not different from the control, but it does respond somewhat to heat stress. There appeared to be a decline (i.e., there was no increase) in CNP activity when the parent strain was heat stressed. It is uncertain why the parent strain did not respond to heat stress, but the behavior of CNP is similar to that seen with GS.

Parker, *et al.*, (1980) reported the CNP activity in unstressed C-6 glioma of cell passage #88 to be  $0.125 \pm 0.009$  umol/min/mg of protein and at cell passage #26 to be  $0.688 \pm 0.032$  umol/min/mg of protein. Results obtained in this laboratory for CNP activity at cell passage #70 in the control cells (PNT) were  $0.207 \pm 0.018$  umol/min/mg of protein. The CNP activity determined in our control cells may be decreasing toward the level found by Parker, *et al.*, at cell passage #88. Based on Parker's data showing a loss of CNP activity with increased passages, these values would be expected at our intermediate passage number.

The activities of CNP, an enzyme marker for oligodendrocytes, and glutamine synthetase, an enzyme marker for astrocytes, were compared. In order to determine whether our cell line was becoming more astroglial in character (undergoing transdifferentiation) our data was compared to the results reported by Parker, *et al.*,

for early (21 to 26) and late (82 to 88) C-6 glioma cell passages. The comparison was undertaken to assess the increase in GS and the decrease in CNP seen with aging (transdifferentiation); although, we only looked at one point in time. Parker, *et. al.*, have indicated that since the CNP activity was markedly high and that of GS was low in the early passages and that this relation was reversed in the late passages; their findings suggested a transdifferentiation of C-6 glioma cells with passage. At our passage #70, the GS/CNP ratio in the control cells was approximately 10:1. Parker, *et. al.*,’s GS/CNP ratio for cell passages #21-26 was approx. 1.5:1. At cell passages #82-86, the GS/CNP ratio was approximately 30:1. The ratio seen after heat stress to the control cells (PNT + HS) was approximately 11:1. The GS/CNP ratio was approximately 10:1 in the colchicine treated cells (CLT) and approximately 11:1 in the heat stressed colchicine treated cells (CLT + HS). Two things are apparent from our data. One is that the cells that we used have GS/CNP ratios that agree fairly well with Parker’s work. The second point is that neither heat shock nor colchicine did much to change this ratio.

Colchicine treatment significantly increased heme oxygenase activity and produced drug resistance. These findings suggest the possibility of using HO as a marker of drug resistance. Neither GS nor CNP activities appear to parallel the induction of drug resistance, and these enzymes are not predictive of the onset of drug resistance.

***Objective III: To determine whether the drug resistance seen in the colchicine treated cells can be reversed by verapamil***

Verapamil presumably acts by inhibiting the activity of the multiple drug receptor and restoring the cytotoxic activity of drugs like adriamycin, vincristine, and taxol within the cell (Dalton, *et. al.*, 1989).

Our studies showed that verapamil, in the absence of taxol, appears to have induced a significant increase in cell counts (approximately 20% over control) when cells (at passage #81) were pretreated with 10uM verapamil (figure 11). However, figure 12 shows that even though cells exposed to 10 uM verapamil showed an increase in cell counts, this concentration still increased the sensitivity of the CLT cell line to taxol. In addition, the lower concentrations that did not appear to affect cell counts (1 uM and 5 uM), also reduced the taxol EC<sub>50</sub> toward that seen in the control cells.

Schmidt, *et. al.*, 1988 studied the effects of verapamil by itself on human medulloblastoma, pinealblastoma, glioma, and neuroblastoma cell lines. They found that growth rates of these tumor cells were inhibited 10-100% by concentrations of 10-100 uM verapamil. Our results indicate that verapamil by itself, does enhance cell counts, at least in the colchicine treated cells, at concentrations up to the lowest value in their study (10 uM).

The fact that the addition of verapamil sensitized our colchicine treated cells to taxol (decreased cell counts) provides further evidence that colchicine has induced multiple drug resistance in this glioma cell line. The ability of verapamil to inhibit the activity of the multiple drug receptor and restore the cytotoxic action of taxol has previously been documented by Dalton, *et. al* (1989).

In addition, our results show that blocking of the MDR receptor by verapamil is concentration dependent. Similar findings have been reported by Twentyman, *et. al.*, 1990 in studies utilizing a panel of multiple drug resistant mouse tumor cell lines. They studied the relationship between resistance to adriamycin, vincristine, colchicine, etoposide and resistance modification by verapamil and cyclosporin A (Table 3). The latter two agents are inhibitors of the multiple drug transporter. When present in excess, these two inhibitors compete for the multiple drug resistance receptor, thereby slowing the disappearance of chemotherapeutic agents from resistant cells.

Twentyman, *et. al.*, (1990) used concentrations of 3.3  $\mu\text{M}$  and 6.6  $\mu\text{M}$  verapamil in multiple drug resistant mouse tumor cell lines. They found that the effect of 3.3  $\mu\text{M}$  verapamil on the MDR cell lines was no greater than that of the parent line. By comparison, studies in our laboratory showed that 1  $\mu\text{M}$  verapamil was effective in sensitizing our colchicine treated cells to taxol. Therefore, it appears that taxol is more effective in our C-6 glioma cell line than in the cell lines studied by Twentyman, *et. al.*, (1990).

Differences were noted in the taxol  $\text{EC}_{50}$  between the colchicine treated cells (1490 ng/ml at cell passage #70) and the colchicine treated cells—no verapamil pretreatment (9103 ng/ml at cell passage #81) (figure 13). These differences in  $\text{EC}_{50}$  values may be a reflection of the differences in cell passage (passage #70 versus passage #81) or the differences may reflect the variation in the times which the two cell passages were harvested. Cell passage #70 was grown to confluence

(approximately 10 days) prior to harvesting. Cell passage #81 was harvested 48 hours after the start of verapamil treatment. It is also possible that taxol may be more effective or have a more substantial affect on cells during the log phase of growth. Therefore, a direct comparison between the two colchicine treated passages is difficult.

A comparison of calculated  $EC_{50}$  values (Fig. 13) demonstrated the effectiveness of verapamil in reversing colchicine induced drug resistance by taxol. The  $EC_{50}$  of taxol was reduced 14-fold; by exposure to 1  $\mu$ M verapamil. At 5  $\mu$ M verapamil pretreatment, the taxol  $EC_{50}$  was diminished 20-fold. The taxol  $EC_{50}$  was decreased 125-fold to 72 ng/ml when cells were pretreated with 10  $\mu$ M verapamil. This  $EC_{50}$  value of 72 ng/ml is comparable to that found with the control cells (taxol  $EC_{50}$ - 82 ng/ml). This indicates that verapamil at a concentration of 10  $\mu$ M completely reversed drug resistance in the colchicine treated cells. Since verapamil is known to inhibit multiple drug resistance, this evidence supports a MDR mechanism for the induction of drug resistance to taxol by colchicine in C-6 glioma.

### **CONCLUSIONS:**

- (1). Colchicine applied for a 24 hour period at a concentration of 480 ng/ml can induce drug resistance to taxol in a C-6 glioma cell line
- (2). This resistance is characterized by:
  - a. An increase in the amount of protein per cell.
  - b. Increased heme oxygenase activity.
  - c. Of the three enzyme tested, only heme oxygenase correlated well with drug resistance
- (3). Colchicine - induced resistance to taxol is reversed by verapamil and therefore is likely due to the induction of the multiple drug resistance receptor.

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APPENDIX A  
CELL CULTURE  
ENZYME ACTIVITY ASSAY  
PROTOCOL

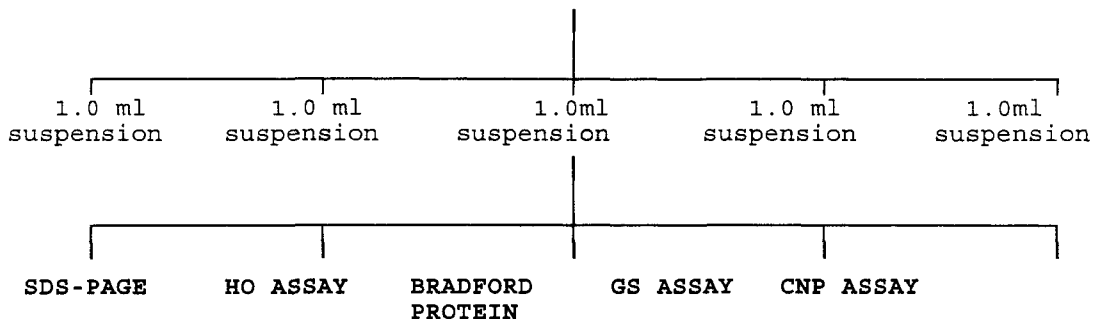
## Cell Culture/ Enzyme Activity Assay Protocol

Harvest cells- 0.25% Trypsin (5.0 ml per 75cm<sup>2</sup> flask;  
1.0 ml per well in 6 well plate; and 0.5 ml per well  
in 24 well plate)

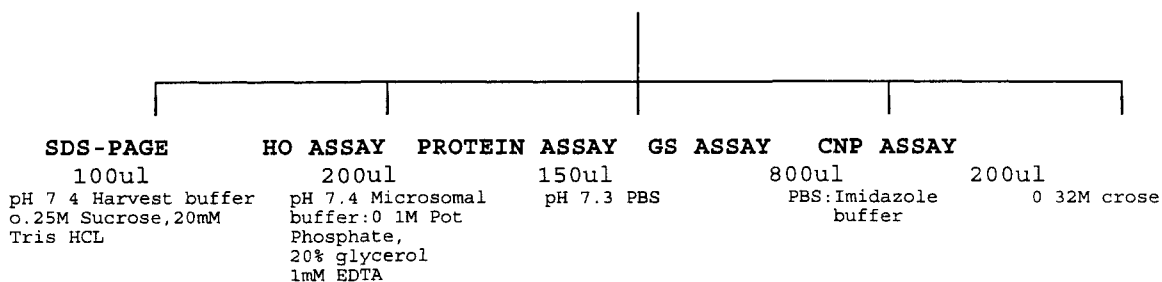
|  
Pipet trypsinized cells in X2- X3 volumes  
ice cold F-12 media

|  
Cell count(s)  
(200ul)

Centrifuge- decant off supernatant media. Re-suspend in 5.0 ml EBBS (Earle's balanced salt solution). Vortex briefly; centrifuge at approximately 2500 rpm for 5 min. Repeat procedure. Finally, re-suspend pellet in 5.0 ml EBBS. Pipet solution into pre-labeled 1.5 ml Eppendorf tubes as follows:



Centrifuge tubes on high setting in Beckman Microfuge for approximately 30 seconds. Decant off supernatant. Re-suspend cells in appropriate volume of buffer as follows



**QUICK-FREEZE** each tube ASAP in liquid nitrogen or dry-ice acetone or isopropanol bath.

**APPENDIX B**  
**ASSAY PROCEDURES**

**CELL COUNTING PROCEDURE**

## Reagents:

Trypan Blue- 0.4% in 0.85% saline  
0.85% sodium chloride (saline)  
Trypsinized cell suspension in cold F-12 media

## Instruments:

Hemocytometer Fisher model  
Olympus model M021 stereomicroscope  
200-1000ul variable volumetric pipettor and tips

## Procedure:

- 1). Into pre-labelled culture tubes the following solutions were pipetted:
  - a. 500ul trypan blue dye
  - b. 300ul 0.85% saline
  - c. 200ul cell suspension in F-12 media
- 2). Each tube was vortexed briefly.
- 3). Using a pasteur pipet transfer sufficient enough volume of cell suspension to fill each grid in a hemacytometer by capillary action.
- 4). All of the cells in each of the four corner and center grids were counted for each sample.
- 5). To obtain the average cell number, the total number of cells counted was divided by the number of grids counted.
- 6). This number was multiplied by 5 to correct for the dilution factor.
- 7). To obtain the number of cells per milliliter in the original solution, the number obtained in step 6 above was multiplied by  $10^4$ .

**BRADFORD PROTEIN ASSAY PROCEDURE**

## Reagents:

- Phosphate buffered saline: pH 7.3 0.01M  
 NaOH solution: 0.05N  
 HCL solution: 1.21N  
 Bovine serum albumin protein standards: 15,30,50 mg/dl in saline.  
 Cell homogenate: suspended in phosphate buffered saline.  
 Protein assay solution: Prepared by mixing 1 volume of protein dye reagent with 9 volumes deionized water in a plastic container.

## Instruments:

- 80°C water bath  
 0-4°C ice bath  
 Bausch & Lomb spectronic 20  
 Eppendorf variable volumetric pipettors with disposable tips  
 vortex mixer

## Procedure:

- 1). 0.1 ml each of protein standards, samples, and distilled water as a blank were hydrolyzed in tubes containing 600 ul 0.05N NaOH by heating at 80°C for 10min.
- 2). After heating the tubes were immediately placed in an ice bath for 5 min.
- 3). The solutions were then neutralized with 1 drop (approx 50ul) of 1.21N HCL.
- 4). 50ul each of the previously hydrolyzed standards, samples and blank were pipeted into tubes containing 2.5ml protein assay solution.
- 5). Each tube was mixed thoroughly by vortexing briefly.
- 6). The tubes were equilibrated at room temperature for 3min. prior to transferring to a cuvette.
- 7). The absorbance [O.D.] of the standards and samples vs the blank were measured at 595nm.
- 8). The amount of protein in each sample was estimated from the slope of the regressed line in which the absorbance [O.D.] was plotted against the concentration (mg/ml) of the protein standards.

**HEME OXYGENASE ASSAY**

## Reagents:

- Potassium phosphate buffer: 0.1M  $K_2HPO_4$ , pH 7.4  
 Deferroxamine: 100mM stock solution, 65.7mg in 1.0ml  $K_2HPO_4$  buffer  
 Bovine serum albumin: 1mM stock solution, 66.0mg in 1.0ml  $K_2HPO_4$  buffer  
 Hemin (Hemin chloride): 1mM stock solution, 6.5mg in 0.1M  $K_2HPO_4$  buffer:0.1N NaOH (9.5:0.5)  
 $\beta$ -Nicotinamide adenine dinucleotide phosphate ( $\beta$ -NADPH): 100mM stock solution, 74.3mg in 1.0ml 0.1M  $K_2HPO_4$  buffer  
 Bilirubin: 1mM stock solution, 5.85mg in 10ml 0.1M  $K_2HPO_4$ : 0.1N NaOH (9.5:0.5)  
 Biliverdin: 1mM stock solution, 5.82mg in 10ml 0.1M  $K_2HPO_4$ :0.1N NaOH (9.5:0.5)  
 Cell homogenate buffer: 0.1M  $K_2HPO_4$ , 20% (v/v) glycerol, 1mM EDTA, pH 7.4  
 C-6 glioma cell homogenate: previously prepared and frozen (see cell culture/ enzyme activity protocol)  
 Rat liver cytosol: previously prepared and frozen, source of biliverdin reductase.

## Instruments:

- 37°C water bath  
 Gilford Stasar III spectrophotomer with temperature controlled flow through cuvette  
 Conical shaped teflon cell homogenizer and drill

## Procedure:

- 1). Cell suspension(s) and one milliliter of rat cytosolic fraction were removed from -20°C freezer and allowed to thaw.
- 2). The cell suspension was homogenized using a teflon homogenizer.
- 3). Into 12 x 75mm culture tubes the following reaction mixtures were pipeted:

<u>Reactant</u>	<u>Amount of Stock Solution to pipet</u>
5mM Deferroxamine	50 ul
25uM Hemin	25 ul
15uM Bovine serum albumin	15 ul
*1mM NADPH	10 ul
C-6 glioma cell homogenate	200ul
Rat cytosolic fraction	50 ul
0.1M $K_2HPO_4$ buffer	650ul
*Note: (see step 4 below)	



- 4). The reaction mixture was preheated without NADPH at 37°C for 5 min.
- 5). 10 ul NADPH was added and the mixture was gently vortexed and then aspirated into the spectrophotometer.
- 6). The absorbance ( $\Delta$ O.D.) was measured at 1 min. and again at 15 min. at a wavelength setting of 453nm.
- 7). The Heme oxygenase activity (nM/min) was estimated by multiplying the rate of change in absorbance ( $\Delta$ O.D./min) by the inverse of the slope of the regression line in which absorbance (O.D.) was plotted against bilirubin standards (nM) :

$$\Delta\text{O.D./min} \times \text{nM/O.D. (inverse slope)} = \text{nM/min}$$

- 8). After correction for the total amount of protein in the original sample volume, Heme oxygenase activity was expressed as pmol/min/mg protein

**GLUTAMINE SYNTHETASE ASSAY****Reagents:**

Lysis buffer: 10mM Imidazol HCL, 0.5mM EDTA, pH 7.0 with HCL  
 Imidazol-HCL: 1M, pH 7.2 with HCL  
 L-glutamine: 0.25M in distilled H<sub>2</sub>O  
 Manganese chloride: 4mM in distilled H<sub>2</sub>O  
 Hydroxylamine: 1.25M in distilled H<sub>2</sub>O, make fresh each time  
 Sodium Arsenate: 200mM in distilled H<sub>2</sub>O  
 Adenosine 5'Diphosphate (ADP): 8mM in distilled H<sub>2</sub>O  
 Ferric chloride: 0.37M FeCl<sub>3</sub>, 0.67M HCL, 0.20M trichloroacetic acid  
 Gamma-glutamyl-hydroxymate (L-glutamic acid gamma-monohydroxymate): 5mM in PBS/lysis buffer  
 Phosphate buffered saline: 0.01M, pH 7.30  
 Previously prepared and frozen aliquot of C-6 glioma cells (see cell culture/ enzyme activity assay protocol)

**Instruments:**

0-4°C ice bath  
 37°C water bath  
 Teflon tissue homogenizer & drill  
 Bausch & Lomb Spectronic 20 spectrophotometer  
 Damon IEC HN-S Centrifuge  
 Vortex

**Procedure:**

- 1). Frozen cell suspensions were thawed and homogenized in PBS/lysis buffer (1:1).
- 2). Tubes were labelled in triplicate for samples, standards, and blanks.

Standard umoles gamma-GH	Vol. of 5mM soln. to add (.005uM/ul)	Vol. of PBS/lysis buffer (ul)
0	0ul	250ul
.02	4	246
.05	10	240
.10	20	230
.20	40	210
.30	60	190
.40	80	170

- 3). To each of the blanks, 250ul of PBS/lysis buffer (1:1) was added.
- 4). The standard curve was set up as follows:
- 5). To the sample tubes, 250ul of cell homogenates were added.
- 6). The reaction mixture was prepared as follows:

	for 40 tubes	for 60 tubes
<b>L-glutamine</b>	4.0ml	6.0ml
<b>Imidazole</b>	2.0ml	3.0ml
<b>ADP</b>	1.0ml	1.5ml
<b>MnCL<sub>2</sub></b>	1.0ml	1.5ml
<b>Hydroxylamin</b>	1.0ml	1.5ml
<b>NaAs</b>	1.0ml	1.5ml

- 7). 250ul of the reaction mixture was added to each tube; blanks, standards, and samples.
- 8). Each tube was vortexed briefly and then placed in a 37°C water bath for 30 minutes.
- 9). After the 30 minute incubation period the tubes were placed in an ice bath.
- 10) 0.5ml FeCL<sub>3</sub> solution was added to each tube, vortexed and then centrifuged at approx. 2000rpm for 5 minutes.
- 11). The FeCL<sub>3</sub> solution containing the gamma-glutamyl-hydroxymate ( $\gamma$ GH) was measured spectrophotometrically at 555nm.
- 12). The absorbance (O.D.) of the FeCL<sub>3</sub> solution for each of the standards and samples were recorded and corrected for the absorbance of the blanks.
- 13). The  $\gamma$ -glutamyl hydroxymate activity (uM/min) was estimated by multiplying the rate of change in absorbance ( $\Delta$ O.D./min) by the inverse of the slope of the regressed line in which absorbance (O.D.) was plotted against varying concentrations (umoles) of  $\gamma$ -glutamyl hydroxymate:

$$\Delta\text{O.D./min} \times \text{uM/O.D. (inverse slope)} = \text{umol/min}$$

- 14). After correction for the total amount of the protein in the original sample volume (800ul), the  $\gamma$ -glutamyl hydroxymate was expressed as umol/min/mg protein.

**2',3'-CYCLIC NUCLEOTIDE 3'-PHOSPHOHYDROLASE (CNP) ASSAY**

## Reagents:

- Sucrose: 0.32M in distilled H<sub>2</sub>O
- Tris-HCL: 0.20M, pH to 7.5
- Sodium Deoxycholate: 1% solution in distilled H<sub>2</sub>O
- Tris-maleate: 50mM, pH to 6.2
- Tris-HCL: 0.30M containing 21mM MgCL<sub>2</sub>, pH 9.0
- 2',3'-cAMP: 7.5mM, in Tris-maleate buffer. Made fresh the day of the assay
- Alkaline phosphatase: approximately 0.7 alkaline phosphatase units/ 100ul of 0.30M Tris-HCL containing 21mM MgCL<sub>2</sub>
- Isobutanol/Benzene: (1:1) mix equal parts of these reagents
- Ammonium molybdate: 1.5% in 0.5N H<sub>2</sub>SO<sub>4</sub>
- H<sub>2</sub>SO<sub>4</sub> solution: 0.5N

## Instruments:

- Teflon tissue homogenizer
- 90°C water bath
- 30°C water bath
- Bausch & Lomb Spectronic 20 spectrophotometer
- 0-4°C ice bath
- C-6 glioma cell suspension, previously isolated and frozen (see cell culture/ enzyme activity assay protocol)
- Beckman Microfuge B

## Procedure:

- 1). Previously frozen aliquots of cell suspensions were thawed and then centrifuged at high speed for 60 seconds in a Beckman microfuge.
- 2). The supernatant was discarded and the resulting pellet was homogenized in 200ul 0.32M sucrose.
- 3). 100ul 0.2M Tris-HCL, pH 7.5 and 200ul 1% Na deoxycholate was added to the homogenate.
- 4). The homogenates were placed in an ice bath for 10 minutes.
- 5). 150ul of distilled water was added to each and then the samples were re-homogenized.
- 6). Using 5ml conical tubes, three blanks were set up by adding 180ul 50mM Tris-maleate, pH 6.2 to each of the tubes.
- 7). Samples were set up in triplicate by adding 130ul 50mM Tris-maleate, pH 6.2 and 50ul homogenate to the appropriate number of tubes.
- 8). 20ul of substrate (7.5mM 2',3'-cAMP) was pipetted into each tube. Each tube was then briefly vortexed.
- 9). The tubes were then placed in a 30°C shaking water bath for 20 minutes.
- 10). After incubation the tubes were first, plunged into a 90°C water bath for 1 minute and then, returned to the

30°C water bath for 5 minutes to cool down.

11). A standard curve for 2'-AMP was set up as follows:

Standard uMoles 2'-AMP	Vol. of 5mM 2'- AMP (0.005uM/ul)	Vol. of Tris- maleate buffer, pH 6.2 (ul)
0	0	200
0.025	5	195
0.050	10	190
0.100	20	180
0.200	40	160
0.250	50	150
0.300	60	140
0.400	80	120
0.500	100	100
0.750	150	50
1.000	200	0

- 12). 100ul of alkaline phosphatase was added to each tube.  
 13). Each tube was vortexed briefly, and then placed in a 30°C water bath for 20 minutes.  
 14). After incubation 1.5ml of isobutanol/benzene (1:1) and 1.5ml of 1.5% ammonium molybdate was added to each tube.  
 15). Each tube was vortexed for approx. 20 seconds and then centrifuged for 5 min. at 2000rpm.  
 16). Color production was measured spectrophotometrically at 410nm.  
 17). The 2',3'-CNP activity (uM/min) was estimated by multiplying the rate of change in absorbance ( $\Delta$ O.D./min.) by the inverse of the slope of the regressed line in which absorbance (O.D.) was plotted against varying concentrations (uMoles) of 2'-AMP standard:

$$\Delta\text{O.D./min} \times \text{umol/O.D. (inverse slope)} = \text{umol/min.}$$

18). After correction for the total amount of protein in the original volume (650ul), the 2',3'-CNP activity was expressed as  $\mu\text{mol}/\text{min}/\text{mg}$  protein.

**RAT LIVER CYTOSOL FRACTION**

## Reagents:

Sodium chloride: 0.15M in distilled water

Tissue buffer: 0.25M sucrose, 20mM Tris-HCL, pH7.4

## Instruments:

Refrigerated centrifuge

Brinkman LJ-65 ultra-centrifuge

Potter-Elvehjem tissue homogenizer

## Procedure:

All preparative steps were performed at 4°C or on ice

- 1). Livers from Sprague-Dawley rats were soaked in approximately 50ml cold 0.15M NaCL.
- 2). Approximately 10gms of diced liver was homogenized in a Potter-Elvehjem homogenizer in 4 vol. tissue buffer, pH7.4.
- 3). The homogenate was then centrifuged at 5,000g for 10min.
- 4). The 5,000g supernatant fraction was then ultra-centrifuged at 24,000 rpm for 1 hour.
- 5). The resultant supernatant cytosolic fraction was then divided into 1.0ml aliquots and immediately frozen in liquid N<sub>2</sub> until needed for the assay procedure.

**SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS  
(SDS-PAGE)**

Reagents:

Stock solution: 30% Acrylamide:0.8% N,N-Methylene bis  
acrylamide (Bis)  
Trisma base  
Tris Running buffer: 1.875M, pH 8.8  
HCL solution: concentrated  
Tris Stacking buffer: 1.0M, pH 6.8  
Ethylenediamine tetraacetic acid (EDTA): 0.2M in distilled  
H<sub>2</sub>O  
N,N,N',N'-tetramethylene amine: TEMED, neat  
Ammonium persulfate: 10% in distilled H<sub>2</sub>O  
Running buffer: pH 8.3, Sigma 7-9 Tris, glycine, Sodium  
dodecyl sulfate (SDS)  
Solubilizing solution: 2x, 10% SDS, 2  $\beta$ -mercaptoethanol,  
glycerol, 1M tris (pH 6.8), distilled H<sub>2</sub>O,  
Bromophenol blue  
Fixer/Destainer: 50% methanol, 7% glacial acetic acid, 43%  
distilled water  
Stain: Coomassie Brilliant Blue R, 0.25% in  
fixer/destainer  
Previously prepared and frozen aliquot(s) of C-6 glioma  
cells  
(see cell culture/ enzyme activity assay protocol)  
Commercially prepared SDS-PAGE molecular weight markers

Instruments:

Extech Model 671 pH Meter  
Lab Line magnetec stirrer  
100°C Oven  
Hoefer Scientific SE600 Vertical Slab Gel Electrophoresis  
Unit  
E-C Model EC600 Power Supply  
Mettler Model 1200 Balance  
50ul Syringe

Procedure:

A. Preparation of the Separating Gel

- 1). The SE600 Vertical Slab Gel Unit was assembled in the casting mode using 1.0mm spacers.
- 2). A 12.5% Separating gel was prepared by pipeting the following solutions, in order, into a 125ml Erlenmeyer flask:
 

a) Acrylamide:BIS (30gm:0.8gm)	12.5ml
b) 1.875M tris, pH 8.8	6.0ml
c) 0.2M EDTA	0.3ml
d) distilled H <sub>2</sub> O	10.9ml
e) TEMED	.015ml
f) 10% NH <sub>4</sub> persulfate	0.3ml
- 3). The solution was immediately pipeted into the sandwiches



to a level about 4.0cm from the top.

- 4). The gel was overlaid gently with approximately 0.3ml distilled water.
- 5). The solution was allowed to polymerize for 20-30 minutes.
- 6). After 30 minutes the overlay was poured off and excess water removed with Whatman no. 1 filter paper.

#### B. Preparation of the Stacking Gel

- 1). The Stacking Gel was prepared by pipeting the following solutions, in order, into a 125ml Erlenmeyer flask:
  - a) Acrylamide:BIS (30gm:0.8gm) 2.5ml
  - b) 1.0M tris, pH 6.8 1.9ml
  - c) 0.2M EDTA 0.15ml
  - d) distilled H<sub>2</sub>O 10.3ml
  - e) TEMED 7.5ul
  - f) 10% NH<sub>4</sub> persulfate 0.15ml
- 2). The 1.0mm spacer comb was inserted into the sandwich.
- 3). The Stacking gel was gently pipeted into the sandwich taking care to remove all air bubbles from around the spacer.
- 4). The Stacking Gel was allowed to polymerize for 20-30 minutes.

#### C. Preparation of Cell Homogenate

- 1). Equal volumes of cell homogenate and 2X Solubilizing Solution were combined in a test tube.
- 2). The tube was placed in a boiling water bath for approximately 90 seconds to denature the proteins with SDS.

#### D. Loading and running the Gels

- 1). The comb(s) were gently removed from the gels.
- 2). Each well was rinsed with Running buffer.
- 3). Using a 50ul syringe the sample(s) (cell homogenates equivalent to 10-20ug protein) and the standard molecular weight markers were underlaid in the appropriate well.
- 4). The Gel Sandwiches were then assembled into the buffer chamber, according to manufacture specifications, and the appropriate volume of Running Buffer, pH 8.3 added.
- 5). The lid was placed on the unit and the unit connected to the power supply. The power supply was run at a constant current of 50 mA
- 6). The gels were electrophoresed with constant cooling and stirring until the marker dye had reached the bottom of plate.

#### E. Staining and Destaining the Gels

- 1). After disassembling the gels from the sandwiches, the gels were fixed in Fixer/Destainer solution for 4 hours

on a rotary shaker.

- 2). The proteins in the gel were visualized by staining with the Coomassie Blue staining solution for 30 minutes and then repeatedly destaining with Fixer/Destainer solution.
- 3). The position of the Heat shock proteins- hsp 32, 70, & 90 were compared by visual comparison to the known molecular weight markers.