Selection Of An ICRF-187-Resistant CHO Cell Line And Measurement Of Cytotoxicity, Additive Cytotoxicity, And Cross-Resistance Of Bisdioxopiperazines, Anthracyclines, And Other Antineoplastic Agents In Wildtype And Resistant Cells

BY

THEODORE I. KUSCHAK

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Faculty of Pharmacy
University of Manitoba
Winnipeg, Manitoba, Canada

ISBN 0-315-99057-0

Name ______________________

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

Health & Environmental Science

~~Cytotoxicity testing of Bis(dioxopiperazines) & Anthracyclines~~

SUBJECT TERM

0 5 7 2

SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION
General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES
American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES
Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- ✗ Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY
General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

Selection Of An ICRF-187-Resistant CHO Cell Line And Measurement Of Cytotoxicity, Additive Cytotoxicity, And Cross-Resistance Of Bisdioxopiperazines, Anthracyclines, And Other Antineoplastic Agents In Wildtype And Resistant Cells

BY

THEODORE I. KUSCHAK

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Abstract

The purpose of this project was to determine the cytotoxicity of a series of bisdioxopiperazines, anthracyclines and other antineoplastic agents. These drugs were tested on a Chinese Hamster Ovary AA8 cell line. The cytotoxicity of the cardioprotective agent, ICRF-187 (dexrazoxane, ADR-529) was also tested in cytotoxicity experiments in combination with a series of cardiotoxic anthracyclines. The purpose of these experiments was to determine whether the ICRF-187 had an antagonistic or additive cytotoxicity. Finally, an ICRF-187-resistant strain of CHO-AA8 cells was selected which allowed for the characterization of bisdioxopiperazine resistance as well as resistance to anthracyclines and other antineoplastic agents.

The important conclusions drawn from this research are as follows: The cytotoxicity of doxorubicin and ICRF-187 to CHO-AA8 is greater than additive. The ICRF-187-resistant cell line, DZR3 attained a resistance factor of 900 fold against ICRF-187. Other bisdioxopiperazines show a similar resistance. The anthracyclines also showed some cross-resistance. DZR3 cells show 13.2 fold and 28.5 fold resistance factors to doxorubicin and idarubicin, respectively, but little to other antineoplastic agents.

This work is dedicated to the memory of my mother,

Louise Elizabeth

Acknowledgments

This page is sincerely dedicated to all those who were an important part of my experiences during my Master of Science Program in the Faculty of Pharmacy.

I thank my supervisor, Dr. Brian Hasinoff for his financial support in the first year of my program. I am most grateful for his guidance and for the skills and perspectives which he has imparted to me during the course of my study in his laboratory. I am grateful to him for his patience and advice during the time when I performed my experiments and certainly, during the editing of this manuscript.

I am grateful to Dr. T.G. Vitti (Faculty of Pharmacy) and Dr. H.B. LéJohn (Department of Microbiology) who provided me with valuable and constructive criticism during the editing of this manuscript.

I wish to pass on my thanks and acknowledge the skills to which I have been introduced by Dr. Hasinoff's skilled technician, Mr. Mukhtiar Singh. I wish also to thank fellow graduate students in Dr. Mike Butler's laboratory, namely Andrew Christie and Mike Berry in the Microbiology Department for their help in getting me started in the art of cell culture.

I am grateful for the advice, friendship and camaradarie of my friend Dr. Yangzhi Ling in the Faculty of Pharmacy.

I acknowledge the financial support of the Health Research Foundation - Pharmaceutical Manufacturers Association of Canada and the Medical Research Council, who have provided me with a two year graduate student scholarship and a travel award.

Finally, I wish to thank my parents for the things they have imparted unto me; my mother, Louise for the desire to learn about the world, my father, Fred for his appreciation of value of education and for the personal sacrifices he has made in order to allow me to study.

To Brenda: I could never ask for a better partner in my life. You have given me a great deal to be grateful for.

Table Of Contents

List Of Figures

List Of Figures

List Of Figures

List Of Figures

List Of Figures

List Of Figures

List Of Figures

List Of Tables

List Of Tables

List Of Tables

List Of Photographs

Chapter 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 A BRIEF INTRODUCTION TO ICRF-187

1.1.1 The Status of ICRF-187

ICRF-187, whose proper chemical name is (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane, is also known as dexrazoxane, and ADR-529. ICRF-187 is a promising new drug currently undergoing multicentre phase III clinical trials in the United States with several hundred patients under treatment. The United States Food and Drug Administration has accorded this drug a "1A" priority status, meaning that it is second in priority only to AIDS drugs. The reason for its status is because of its novel effects and most importantly, because of its strong therapeutic life saving effect. In spite of the fact that this is a drug which is considered to be in the late stages of development, there is little known about its chemistry or biochemistry.

1.1.2 The Compound and Its Nature

ICRF-187 is a small, uncharged membrane permeable compound. At physiologic pH and temperature, it undergoes slow ring-opening hydrolysis with a half-time of reaction of approximately 9.3 hours (Hasinoff, 1993). The hydrolysis of ICRF-187 from its initial rings-closed form, (shown in figure 1.4.1.1) to its rings-open form, ADR-925, enables it to remove 100% of the iron from transferrin (Hasinoff and Kala, 1993). In its rings-open form it resembles the strong chelating agent EDTA (Huang *et al.*, 1982). It is a strong chelator of copper and iron like EDTA. However, it possesses a single advantage as a biological chelator, which is not found in EDTA. The EDTA compound is highly

polar and consequently is not membrane permeable. ICRF-159 (razoxane), which is the racemate of ICRF-186 and ICRF-187 has been shown to diffuse into cultured cells (Dawson, 1975). ICRF-187 in its rings closed form is in effect a prodrug able to enter a cell as a non-polar compound. Upon hydrolysis it becomes a strong chelating agent able to function inside the cell to efficiently remove iron from the Fe^{3+}-doxorubicin complex, (Hasinoff 1989, 1990).

1.2 THE PROBLEM

A large body of evidence indicates that one of the greatest mediators in a variety of different types of tissue damage is the oxygen-derived free radical. This usually occurs as a result of metabolism of chemicals and drugs, as well as in ischemic damage such as that following heart attack and stroke. For example, doxorubicin is limited in its usefulness as an antitumor drug by a unique and potentially fatal cumulative dose-dependent cardiotoxicity (Gianni *et al.*, 1983). Doxorubicin doses recommended in the Compendium of Pharmaceutical Specialties (1994) are limited to 550 mg m^{-2} for the 21 day regimen and 700 mg m^{-2} of body surface area for the weekly regimen. The damage inflicted on the heart is believed to occur through the generation of hydroxyl free radicals (Halliwell and Gutteridge, 1985). Damage caused by free radicals is also known to occur as a result of radiation injury, inflammation and degenerative diseases such as arthritis and aging (Fantone and Ward, 1985; Halliwell and Gutteridge, 1985; Halliwell and Gutteridge, 1986; Gianni *et al.*, 1983; Demant and Nørskov-Lauritsen, 1984; Demant, 1986; Braughler and Hall, 1989).

The common ingredient in all of the above mentioned conditions is the oxygen free radical. These radicals can be produced naturally in biological systems. Examples of naturally produced oxygen free radicals include hydrogen peroxide, superoxide and the hydroxyl radical. Hydrogen peroxide is produced in many aerobic cells *in vivo* and reaction of H_2O_2 with iron is a feasible source of hydroxyl radicals. The excessive production of free radicals usually accompanies tissue reperfusion with oxygen following a stroke or a heart attack. The body's normal defences are overwhelmed by the overproduction of these radicals and tissue damage results (Halliwell and Gutteridge, 1985).

1.2.1 The Chemistry Behind The Problem

The production of strongly oxidizing hydroxyl radicals is believed to occur naturally in most biological systems through an iron-catalyzed Haber-Weiss reaction (Fantone and Ward, 1985; Halliwell and Gutteridge, 1985; Halliwell and Gutteridge, 1986). The following is a simplified sequence of reactions:

$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$ (formation of hydrogen peroxide) [1]

$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$ (reduction of Fe^{3+}) [2]

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$ (Fenton reaction) [3]

The production of the $O_2^{\bullet-}$ might result from several biological reactions. It can be generated by the action of xanthine oxidase on xanthine, by arachadonic acid metabolism in the cyclooxygenase pathway, autooxidation of catecholamines and oxy hemoglobin, mitochondrial leakage of $O_2^{\bullet-}$ in the respiratory chain or from the respiratory burst associated with neutrophil activation resulting from tissue injury.

1.2.2 The Biological Consequences Of Free Radical Generation

The hydroxyl free radical is a reactive species and it is free to initiate lipid peroxidation as well as damage to cellular organelles. The initiation of lipid peroxidation is thought to occur through the following reactions beginning with the allylic hydrogen of an unsaturated fatty acid (LH) (Halliwell and Gutteridge, 1985).

$$LH + OH^{\bullet} \rightarrow L^{\bullet} + H_2O$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

Exposure of cells to ionizing radiation in the form of X-rays or γ-radiation can result in radical production. Oxygen free radicals are also known to be responsible for a large portion of DNA damage, especially that caused by ionizing radiation. Single and double strand breaks in DNA are considered important events, where DNA damage is concerned. The double stranded breaks are especially critical, since they cannot be repaired. Sufficient DNA damage within a cell may even result in a suicide response by the cell. Since DNA repair is not totally efficient, an extensively damaged cell unable to repair its DNA may destroy itself in order to prevent the reproduction of mutated cells (Halliwell and Gutteridge, 1985).

1.2.3 Iron And Its Place In Biological Free Radical Chemistry

The concentration of free iron in biological systems is very low (Halliwell and Gutteridge, 1985). However, Halliwell and Gutteridge (1986) have detailed potential

sources of iron in the biological system. Phosphate complexes (*i.e.* ATP, ADP, GTP), citrate, carbohydrates and organic acids, DNA, and membrane lipids are all able to loosely bind iron. Sources of tightly bound non-heme iron are iron bound to proteins such as ferritin, transferrin, lactoferrin, and hemosiderin. The action of proteases on hemoglobin following reperfusion injury may also result in the release of iron (Braughler and Hall, 1989). The majority of the body's iron is bound in various enzymes, hemoglobin and transferrin. The remainder, not required for any specific functions, is stored in ferritin. Free iron has been shown to be liberated from ferritin or from the degradation of heme proteins during times of oxidative stress. The release of iron is not problematic in itself. It is the potential of the iron to complex with other complex molecules that is a source of danger to the body and its defences.

On a clinical level, circulating serum iron has been shown to increase the risk of acute myocardial infarction in men (Solonen *et al.*,1991). Iron loading in rats was shown to increase the susceptibility to oxygen perfusion damage, and a number of experiments have shown that the removal of iron by chelation therapy can prevent experimental ischemic myocardial injury and prevent post ischemic lipid peroxidation in rats (van der Kraaij *et al.*, 1988). An increasing body of evidence suggests that the toxicity exhibited by the Fe^{3+}-$(doxorubicin)_3$ complex may be due to an iron based Fenton-type reaction such as is shown by reaction [3] in section 1.2.1.

Under most circumstances, the body is equipped to deal with the generation of free radicals. It is equipped with a number of enzymes such as superoxide dismutase, catalase,

and glutathione peroxidase, which are able to protect the body from the harmful effects of hydroxyl free radicals (Halliwell and Gutteridge, 1985). Under oxidative stress endured by patients undergoing chemotherapy, the level of oxygen free radicals is likely to increase as is the damage to organs, especially those which have low or virtually nonexistent enzymic defences against free radicals. The heart is most susceptible to oxidative stress since heart tissue lacks the protective enzymes found in well protected organs such as the liver and kidneys. Doxorubicin has been shown to complex with iron in a three iron to one doxorubicin ratio (Myers *et al.*, 1982; Beraldo *et al.*, 1985), which has been shown to generate hydroxyl free radicals (Doroshow, 1983). When the body is subjected to an oxidative stress, the enzymic system is overwhelmed and the organ most like to suffer is the heart, since it is poorly equipped to deal with this stress. In order to help in defending the heart or any other organ from these stresses, the chemistry of these oxidative stresses must be understood.

1.2.4 Anthracyclines Are Powerful, But Dose-Limited Chemotherapy Drugs

Anthracyclines, most notably doxorubicin (trade name Adriamycin) are potent antitumor compounds able to damage cells through a variety of mechanisms. One mechanism involves strong DNA binding, which is characteristic of doxorubicin and daunorubicin (Gabbay *et al.*, 1976). The planar nature of the anthracycline molecule enables it to intercalate into DNA and affect replication by interfering with the spatial arrangement of the DNA, and sterically hindering nuclear protein function. Doxorubicin

is not like other intercalating agents (*i.e.* ethidium bromide) which simply bind to DNA. It was shown that doxorubicin was able to induce protein-associated DNA breaks (Rowe *et al.*, 1985). The second mechanisms by which anthracyclines are able to damage cells is through inhibition of topoisomerase II activity. Doxorubicin was shown to inhibit DNA replication and cause topoisomerase II-mediated DNA strand breakage even at low concentrations (Tewey *et al.*, 1984).

Anthracyclines are also known to undergo other reactions such as one and two electron reduction. These reactions trigger a series of reactions generating a number of highly reactive and potentially cytotoxic species. One such reaction is the one electron reduction of doxorubicin to a semiquinone. (Figure 1.2.4.1). The reaction can occur through the activity of a number of enzymes (*i.e.* xanthine oxidase, cytochrome P450 reductase, b5 reductase, and NADH dehydrogenase) (Abella and Fisher, 1984; Lown, 1985). In the subsequent reaction, the semiquinone can react rapidly with oxygen (rate constant of 10^8 M^{-1} sec^{-1}). In tissues where superoxide dismutase levels are in low concentration, $O_2^{\bullet-}$ chemically dismutates at a considerably lower rate. In this instance, H_2O_2 can then react with a number of species to form $OH^{\bullet}$. It is this product which is available to react with a variety of biomolecules, including DNA, RNA, cell membranes and several proteins, causing widespread damage and cell death. It is for this reason that there is a great deal of attention given to the reaction mechanisms of anthracyclines, which are able to generate $OH^{\bullet}$ (Kalyanaraman, *et al.*, 1984; Muindi *et al.*, 1984; Mimnaugh *et al.*, 1983; Doroshow, 1983).

Among the reactions that occur with doxorubicin, only one reaction is metal-dependent: that is the direct reaction between the Fe^{2+}-doxorubicin and H_2O_2. There are two broad classes of metal ion dependent pathways. The first involves the reduction of either Cu(II) to Cu(I) or Fe(III) to Fe(II), either by the drug semiquinone or by $O_2^{\bullet-}$ (Reaction [6]). The second mechanism involves a direct reaction between the doxorubicin-Fe(II) complex and H_2O_2 (Reaction [7]). These reactions are listed in Table 1.2.4.1.

Doxorubicin One Electron Redox Cycling

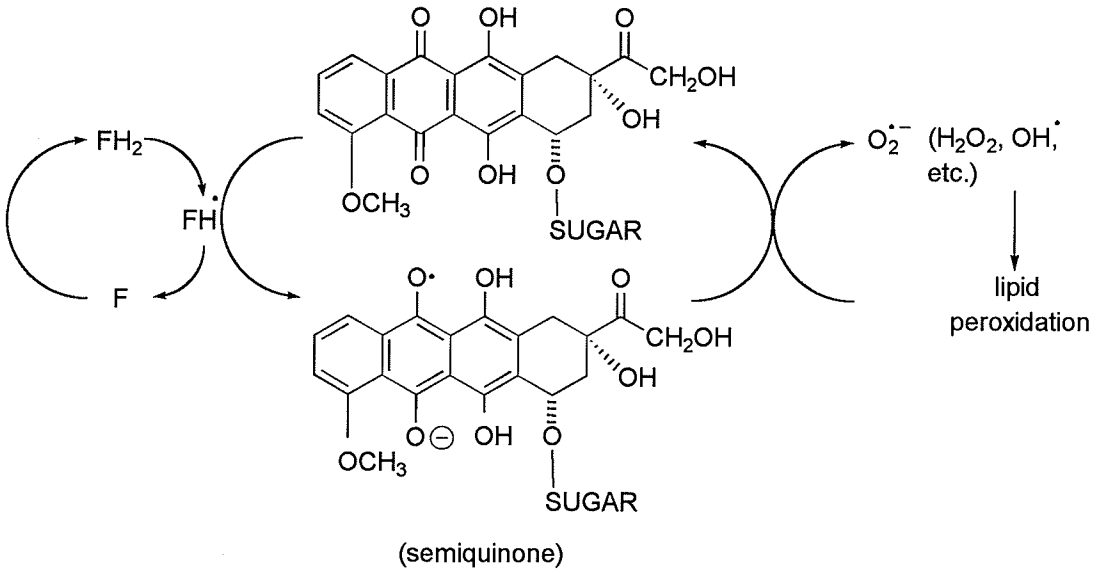


Figure 1.2.4.1 (Modified from Keizer *et al.*, 1990)

Table 1.2.4.1

Formation Of $OH^{\bullet}$ By Doxorubicin

$DOX^{\bullet-} + O_2 \rightarrow DOX + O_2^{\bullet-}$ (enzymic activation) [4]

$2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$ (dismutation) [5]

$O_2^- + Fe(III) \rightarrow O_2 + Fe(II)$ [6]

$DOX + Fe(III) \rightarrow DOX(oxidized) + Fe(II)$ [7]

$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^{\bullet} + OH^-$ [8]

As stated above, most of the iron is tightly bound to proteins such as transferrin or ferritin, or incorporated into porphyrins. In circulation, the iron is stored bound to transferrin, which has its own receptors on the surface of cells. Upon binding a transferrin receptor, the transferrin molecule is internalized in small vesicular structure (Hunt and Marshall-Carlson, 1986). The pH of these vesicles is quickly acidified to between 5.5 and 5.6, at which point the iron is released from the protein and leaves the vesicle. Subsequently, it is either stored in ferritin or incorporated into various enzymes or cytochromes. Doxorubicin has been shown by Demant *et al.* (1986) to chelate the iron that is bound to ferritin when the pH drops to 6. Semiquinone formation by doxorubicin under anaerobic conditions results in the rapid release of iron from ferritin, which is the major intracellular storage site for the metal ion. Thus, as has been described, doxorubicin is able to acquire iron from two major *in vivo* storage sites and the total iron available from these sites can be as high as 10^{-4} M (Bezkorovainy, 1980). It is not likely that the doxorubicin free radical chemistry will be limited by a shortage of iron.

Doxorubicin is known to complex with iron in a 3-drug to one Fe^{3+} arrangement Gianni *et al.*, 1983). The Fe^{3+}-$(doxorubicin)_3$ has a stability constant of 10^{33} and stepwise stability constants of 10^{18}, 10^{11}, and $10^{4.4}$ (May *et al.*, 1980). Previous work has shown that this complex is responsible for cytochrome *c* oxidase inhibition (Hasinoff, 1989) and inactivation (Hasinoff, 1988, 1989).

It is because of the Fenton-type reactions involving anthracyclines, (reaction [3]) discussed earlier, that doxorubicin is cardiotoxic. These properties are useful in the killing of tumors, but the problem of extreme toxicity to healthy cells arises. Protection of the organs most susceptible to free radical damage would allow for the effective eradication of tumors with minimal damage to the patients healthy cells, especially the heart.

1.3 ANTIOXIDANTS AS A SOLUTION TO THE FREE RADICAL PROBLEM

Due to the reactions discussed above, it can be safely assumed that the body is in constant exposure to hydrogen peroxide and other reactive oxygen species formed throughout numerous enzymic reactions (Fridovich, 1975; Chance *et al.*, 1979). The majority of these oxygen species are not only toxic (Halliwell and Gutteridge, 1984), but they have been shown to be potentially mutagenic (Moody and Hassan, 1982; Cunningham and Lokesh, 1983) and carcinogenic (Ito *et al.*, 1981). Several defensive systems in the form of enzymic detoxication mechanisms are in place to deal with the deleterious effects of the oxygen free radicals (Hassan and Fridovich 1980). A number of biological antioxidant compounds are also shown to act in minimizing the harmful effects

of free radicals. The naturally occurring antioxidants include ascorbic acid (vitamin C), glutathione, α-tocopherol (vitamin E), β-carotene and uric acid (Andrae *et al.*, 1985). Investigators using cell culture models are showing that other compounds are capable of ameliorating the damage mediated by oxygen free radicals (Andrae *et al.*, 1985).

Though these are effective antioxidants they may not be sufficiently powerful tools to deal with the effects of antitumor therapy and heart damage. In the cases where a tumor can be dealt with swiftly using an anthracycline therapy, the alternative is to target an element in the pathway leading to the generation of free radicals rather than trapping the radicals themselves. There are several potential approaches to stemming the production of free radicals. The i*n vivo* use of superoxide dismutase which catalyzes reaction [1] (see section 1.2.1) in combination with catalase, which catalyses the decomposition of H_2O_2 and prevents the completion of reactions [2] and [3] was limited by the fact that the two enzymes are proteins. Being proteins, they can act only outside the cell, and are unavailable for the protection of the internal compartments of the cell where enzyme and DNA damage are occurring (Greenwald, 1990).

Another approach is to target reaction [3] by temporary depletion of the iron available to the cell. The reduction of the available iron to a cell can be accomplished by a suitable chelating agent which is able to pass through the cell membrane to perform its function. A group of compounds ideally suited for the task of entering the cell and temporarily sequestering the cell's available iron are the bisdioxopiperazines and most notably, ICRF-187.

1.4 THE CHEMICAL NATURE AND THERAPEUTIC VALUE OF ICRF-187

1.4.1 The Biochemistry of ICRF-187

In considering the overall activity of ICRF-187, its activities must be examined from the time it becomes available to the cell. ICRF-187 is a non-polar uncharged compound able to traverse the cell membrane as long as is remains in its rings-closed form (Figure 1.4.1.1) (Hasinoff and Kala, 1993). Once opened, it becomes negatively charged and increasingly polar as each of the two rings is hydrolyzed. In solutions which are of physiologic pH and temperature, ICRF-187 will begin to hydrolyze to its rings-open form with a $t_{½}$ of 9.3 hours (Hasinoff, 1993). ICRF-187 must enter the cell as a rings-closed molecule (*i.e.* prior to base-calatyzed hydrolysis) since once hydrolyzed, it is unlikely to be able to enter the cell.

Inside the cell, and under non-saturating conditions, the enzyme dihydropyrimidine amidohydrolase (EC 3.5.2.2 or DHPase), acts on the drug to open one of the rings of both ICRF-187 and its (-)-(*R*) isomer ICRF-186, but at different rates (Hasinoff, 1993). DHPase is present in the liver and the kidney, but is lacking in the heart (Hasinoff, 1991). The ability of DHPase to catalyze the hydrolysis on ICRF-187 is comparable to the rate at which it hydrolyzes its natural substrate, dihydrouracil. (DHPase under non-saturating conditions does however, act on ICRF-187 approximately four times faster than it does on ICRF-186). The single-ring hydrolysis products of both ICRF-186 and ICRF-187 catalyzed by DHPase are no longer substrates for the DHPase enzyme. The second ring is likely opened by base-catalyzed hydrolysis. This may explain the difference in the rate

of ring opening, even though the $t_{½}$ of opening of the second ring is halved by the enzymic opening of the first ring.

Hasinoff (1992) has shown that the hydrolysis of ICRF-187 must proceed through either of two intermediate forms, **B** and **C**, prior to its final degradation to ADR-925 Figure 1.4.1.1). ICRF-187 is lost from the reaction mixture with a half-life of 9.3 hours, but ADR-925 is produced with a half-life of 23 hours. The ring closest to the methyl group is able to open 2.1 times faster than the other ring. Thus it has been shown that 68% of the ADR-925 product is produced via degradation through the **B** intermediate and 32% is produced through the **C** intermediate. **B** is produced more rapidly than **C**, but it is known to accumulate and degrade more slowly to ADR-925 than does the **C** intermediate.

The opening of the bisdioxopiperazine ring is the transition of the ICRF-187 molecule from the status of prodrug to an active metal-chelating drug, known as ADR-925.

1.4.2 The Metal-Chelating Chemistry Of ICRF-187

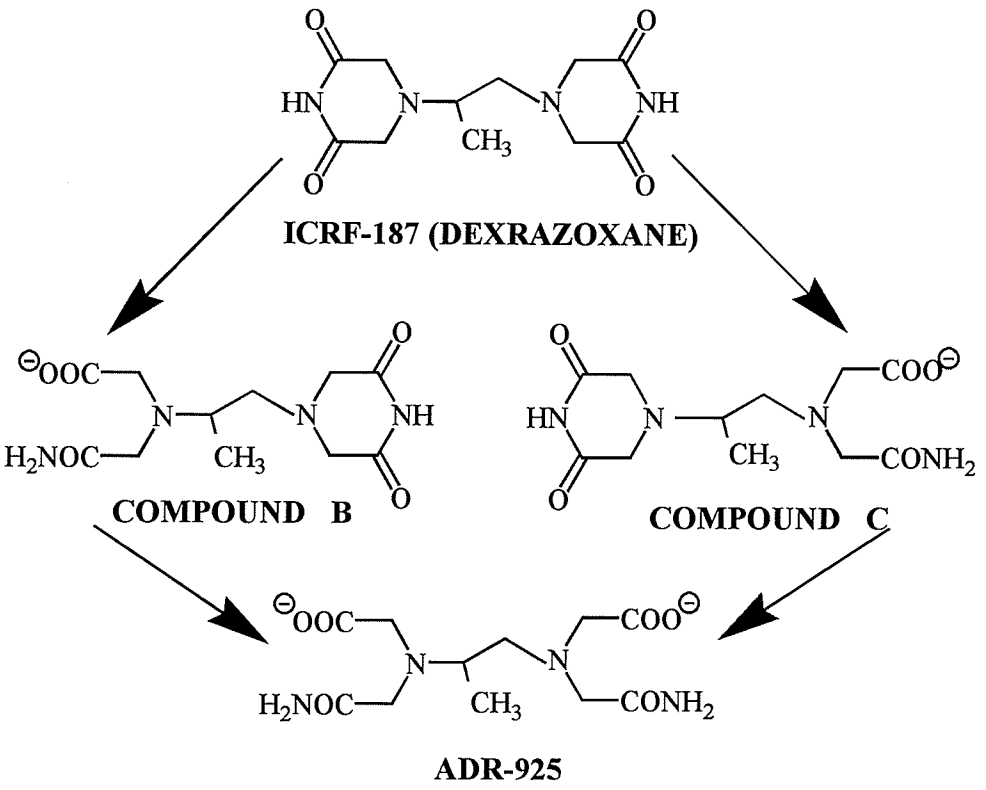
1.4.2.1 Removal Of Iron From Transferrin and Ferritin

In vivo, iron may be released from transferrin and ferritin by acidic conditions by receptor-mediated endocytosis (Morley and Bezkorovainy, 1985; Van Renswoude *et al.*, 1982; and Yamashiro *et al.*, 1984). ADR-925 was able to remove iron from these proteins as well, but these reactions were strongly pH-dependent, occurring at significant rates only when the pH was below 6.5 (Hasinoff and Kala, 1993). If the membrane permeable ICRF-

187 was able to enter the cell through the membrane and then somehow undergo hydrolysis in the acidic environment, it might then be able to remove iron from transferrin (Hasinoff and Kala, 1993). Doxorubicin has been shown to remove iron from transferrin under acidic conditions at rates similar to those seen for ADR-925. Since ADR-925 is able to remove iron from iron-doxorubicin complexes (Hasinoff, 1989, 1990), it should also be able to remove transferrin-derived iron from iron-doxorubicin complexes.

Figure 1.4.1.1

ICRF-187 (DEXRAZOXANE), COMPOUNDS B, C, AND ADR-925



1.4.2.2 Removal Of Iron From The (Doxorubicin)$_3$-Fe^{3+}- Complex

Once in its rings-open state, ADR-925 is able to easily chelate free and very stable anthracycline-complexed iron. In its prodrug form as ICRF-187 was shown to displace iron from the anthracycline complex very slowly ($t_{½}$~30 min.). ADR-925 was able to chelate iron from the Fe^{3+}-(doxorubicin)$_3$ complex, displacing the iron completely and very rapidly ($t_{½}$~1 min.). The overall reaction is as follows:

$$Fe^{3+}\text{-DOX} + \text{ICRF-187} \rightarrow Fe^{3+}\text{-(ADR-925)} + \text{DOX (hydrolysis)} \quad [9]$$

Sequestering of free iron as well as removal of iron from the doxorubicin-iron complex, is likely to affect the anthracycline-catalyzed production of hydroxyl free radicals. Prevention of this reaction is likely to interrupt the formation of the free radicals.

1.4.3 The Cytotoxicity Of ICRF-187

Cytotoxicity experiments conducted with the racemate of ICRF-187, ICRF-159, show that, in addition to the metal ion chelating capacity of these agents, there are also cytotoxic properties. Creighton *et al.*, (1979) showed that ICRF-159 as well as the two isomers comprising it, ICRF-187 and ICRF-186 show cytotoxic effects with cells incubated *in vitro*. IC_{50} values for each of these was shown to be equivalent, *i.e.* 3.0 μM.

1.4.4 Interaction Of ICRF-187 With Topoisomerase II

Bisdioxopiperazines have been shown to be cytotoxic by a number of investigators (Ishimi *et al.*, 1992; Narita *et al.*, 1990; Tanabe, *et al.*, 1991; Ishida *et al.*,1991; Gorbsky,

1994) each of whom has suggested that the toxicity mediated by these agents was directed primarily at a DNA unwinding enzyme known as topoisomerase II. Unlike intercalating drugs, which induce breaks in the DNA by stressing the chain, and other topoisomerase II targeting drugs, which form cleavable complexes, the bisdioxopiperazine compounds inhibit the enzyme directly prior to the formation of the intermediate cleavable complexes in the cell cycle (Tanabe *et al.*,1991).

Studies conducted by Creighton *et al.*, (1979) showed that addition of ICRF-159 to a synchronously growing culture of cells results in the arrest of the growth of the cells at the G_2/M mitosis phase boundary. In considering the ramifications of interrupting topoisomerase II activity, the observations made by Creighton make sense. The role of topoisomerase II in terms of mitosis is to decatenate the daughter strands of DNA prior to separation of the cells. Interruption of the process leaves the two daughter cells unable to separate (Ishimi *et al.*, 1992).

1.4.5 The Potential Of ICRF-187 In Antitumor Therapy

In a careful animal study, Herman *et al.*, (1985) conducted experiments with a series of structurally related bisdioxopiperazine analogues, where the purpose of the experiment was to measure the protective capacity of these analogues against acute daunorubicin toxicity. Single doses of daunorubicin delivered to Syrian golden hamsters (25 mg kg^{-1}) resulted in a loss of body weight and eventual death in 84% of the animals within 1 to 4 weeks. Pretreatment of the hamsters with ICRF-187 was shown to reduce

the lethal effects of daunorubicin. Over 70% of the hamsters were alive at 8 weeks. The same results were obtained with ICRF-186, indicating that the effects are not stereospecific. Since daunorubicin, like doxorubicin is able to affect cells in different ways, it is not known whether ICRF-187 was interrupting DNA interaction or the oxidative damage of daunorubicin by free radical generation. Myers *et al.*, (1982) showed that doxorubicin-iron complexes were able to generate hydroxyl free radicals and seriously damage erythrocyte ghost membranes. When the same experiment was done, but with pretreatment with EDTA, which is structurally and functionally similar to the ICRF-187 hydrolysis product, ADR-925, the pretreatment was shown to prevent membrane damage to erythrocyte ghost membranes (Myers *et al.*, 1982). Clinical trials conducted by Speyer *et al.*, (1992) showed that administration of doxorubicin with ICRF-187 allowed for the delivery of doxorubicin doses as high as 2,000 mg m^{-2} of body surface area to enhance antitumor activity, but with minimal cardiotoxicity. This represents a 3.6 fold increase in the dosage of doxorubicin that can be delivered when administered with ICRF-187.

1.5 RESEARCH CONDUCTED AND REPORTED ON IN THIS THESIS

The topics covered in the course of this project are based entirely on the above discussion of the anthracycline-induced toxicity problems and potential solutions offered by iron chelation as observed in *in vitro* and *in vivo* antitumor therapy studies. This project was conducted entirely on an *in vitro* cell culture model. The goal of this project was to gain insight into anthracycline toxicity, the protective and/or cytotoxic effects of

bisdioxopiperazines, and finally to gain an understanding of the resistance and possible cross-resistance mechanisms developed in an ICRF-187-resistant cell line.

More specifically, Chapter 2 covers the preliminary elements of cell culture, selection of cells, end-point detection, chemicals and equipment used, preparation of solutions and drugs, biohazard certification, cryogenic storage and thawing of cells, etc. Chapter 3 covers single-drug cytotoxicity experiments involving bisdioxopiperazines. Chapter 4 covers the cytotoxicity experiments of anthracyclines coupled with ICRF-187 or its final metabolic product, ADR-925 as well as other bisdioxopiperazines. Chapter 5 covers the selection and isolation of an ICRF-187-resistant cell line, its characterization and testing for cross resistance to other antitumor agents.

Chapter 2

PREPARATION FOR CYTOTOXICITY TESTING

2.1 THE ESSENTIALS OF CELL CULTURE EXPERIMENTS

2.1.1 Selection of the Appropriate Mammalian Cell Line for Cytotoxicity Experiments Involving Anthracyclines and Bisdioxopiperazines

Though a great deal of work is underway in the testing of ICRF-187 at the clinical level, much less is known regarding its mechanisms at the biochemical level. Cell culture methods were employed to investigate these phenomena. The selection of a cell line upon which the drugs would be tested was a matter of central importance to the project.

The selection of an appropriate cell line was approached from several perspectives and many questions were weighed in the course of selection the process. The questions of longevity of the cell line as well as the problems of retaining cellular differentiation and function were considered. The use of primary cell cultures, *i.e.* those derived from freshly sacrificed animals was discounted for several reasons, the most important of which was the initial inexperience with cell culture methods, especially those concerning the isolation and propagation of a pure primary culture. Also, the 50-passage time frame of retention of differential functions after the initial isolation of primary cells would make the cells difficult to work with since the cells would have to be used within a very narrow window of passages in order to ensure the identical level of cell function and differentiation during the experimental phase.

A more practical approach appeared to be the purchase of an established and well characterized cell line from a cell bank such as the American Type Culture Collection. The choices of cells was made from two major categories, namely 'normal' and

transformed cells. The typical characteristics of normal animal cells are outlined by Hayflick and Moorehead, (1961), who studied a normal human embryonic tissue designated WI-38. These characteristics included anchorage-dependence, density inhibition and confluent monolayer cultures as a growth control, a finite life-span (usually 50 passages), a diploid chromosomal compliment, and non-malignancy (shown by the cells' inability to form tumors). The category of cells known as transformed cells have acquired the capacity of infinite growth and for this reason they are often referred to as a continuous or immortal cell line. The transformation process usually results in the cells' loss of sensitivity to the numerous signals usually associated with the growth process. This is exemplified by loss of both anchorage dependence and density inhibition. Other changes include changes in the chromosome patterns (Butler, 1981).

For cytotoxicity studies, it is desirable to have a well understood and characterized cell line that does not contribute inconsistencies to the experiment. Among the common choices used in research and production are BHK-21, HeLa, CHO-K1, MRC-5, SCLC, V79-4, and Vero to name only a few.

In order to determine the best cell line for toxicity experiments, an extensive literature search was conducted, bearing in mind the application to tumor research. Several continuous lines were found to be used in this type of work, but the following were found to be used most often: MCF-7 (a human breast adenocarcinoma), Chinese Hamster Ovary AA8 cells (derived from CHO-K1), and V79-4 (a Chinese hamster lung fibroblast cell). The characteristics of each of these cells is outlined in the *ATCC Catalogue of Cell Lines*

and Hybridomas (1992).

Each of the above-mentioned cell lines has been utilized in drug experiments, be it for single or multiple drug effects, drug resistant cell studies and cross-resistance studies. V79 cells have been used to measure the protective effects of pyruvate and other α-ketoacids from the cytotoxic effects of hydrogen peroxide by Andrae *et al.* in 1985. Protective effects by natural polyphenols against hydrogen peroxide damage were measured by Nakayama *et al.* in 1992, and the role of antioxidants in protection against oxidative stress-induced DNA damage was examined in two V79 clones by Martins *et al.* in 1991. The above mentioned constitute but a small number of the examples found where V79 cells or modified V79 clones were used in various types of cytotoxicity experiments. More frequently used were Chinese Hamster Ovary Cells. Various strains were utilized, among them CHO-K1, CHO-AA8, CHO-UV41, CHO-EM9 and others. CHO cells, specifically AA8, were implicated in many diverse types of research involving hyperoxia and hydrogen peroxide effects on NAD(H) and ATP pools (Gille *et al.*, 1989), UV light effects on sister chromatid exchanges, replicon initiation and thymidine incorporation (Taft *et al.*, 1991), protection against hydrogen peroxide induced DNA damage and cytotoxicity of the calcium chelator Quin 2 (Cantoni *et al.*, 1989) and excision repair mutant isolation to measure Mitomycin C bioactivation (Dulhanty *et al.*, 1989). In pharmaceutical applications, CHO cells have been used by O'Brien *et al.*, (1992) to measure the potential antileukemic activity of a platinum derivative CI-937 and Wilson *et al.*, (1989) used CHO-AA8 cells to measure the effects of hypoxia-selective antitumor agents. The

noteworthy characteristic of CHO-AA8 is that it is a DNA repair mutant which shows sensitivity to some methylating and ethylating agents as well as a number of compounds which are capable of inducing DNA single strand breaks. (Wilson *et al.*, 1989) Once the DNA of the cell has been damaged, it is unlikely to repair the damage, and so damage inflicted during a toxicity experiment would be detected, rather than being masked by a repair mechanism.

Because of its use by many investigators in cytotoxicity, hypoxia, and DNA damage experiments, as well as its DNA repair deficiency, CHO-AA8 was selected as the initial cell line for this project. CHO-AA8 has a doubling time of 12 hours, grows as a monolayer or in suspension. (ATCC, 1992). The most obvious question regarding the choice of the CHO-AA8 cells line for cytotoxicity experiments is why this or any other non-human cells line was selected over a human cell line such as MCF-7, for example. The CHO cells were used in a number of diverse types of experiments such as cytotoxicity, hyperoxia experiments, lactate dehydrogenase assays as well as a number of molecular biology experiments related to cancer therapy. While MCF-7 is an excellent human cancer cell line for measuring the effects of antitumor drugs, it is not a good "all purpose" cell with sufficient versitility for different types of experiments to be conducted the laboratory in the future.

2.1.2 Selection of the Optimal Serum Supplement for the αMEM Used to Grow Chinese Hamster Ovary AA8 Cells

2.1.2.1 INTRODUCTION

Once the appropriate cell line for the research to be conducted has been selected comes, the correct culture medium must be purchased. The choices of medium supplements necessary for CHO-AA8 cells was found by consulting the ATCC which provides the appropriate growth medium, supplements, hormones, etc. required for growth of all types of cells. The use of Alpha Minimal Essential Medium (αMEM) is recommended with supplements of fetal calf serum 10% (v/v). The characteristics of this cell culture medium are described in 2.2.2 and its preparation for use is described in 2.3.1.

A comparison of the growth characteristics of CHO-AA8 cells was made in order to determine which calf serum supplement would be appropriate for the research to be conducted. The recommended calf serum supplement, according to ATCC was a fetal calf serum. Questions regarding the actual benefits of this serum type on growth as well as its prohibitive expense arose. It was important to determine whether the fetal serum, with its increased cost actually possessed qualities that were superior, or desirable for the research to be conducted in our facility.

A growth characteristic experiment was initiated to determine whether the choice of serum supplement added to the cell culture medium had any substantial effect on the growth of the CHO-AA8 cells. The end point was simply to determine the rate of growth as well as the ratio of cells anchored to those freely floating as a result of using the

different sera.

The serum supplements were as follows: The first serum was an iron enriched and supplemented calf serum, added to the medium to comprise 10% (v/v). The second serum type was an iron enriched and supplemented fetal serum added to the medium to give 10% (v/v). The third was a mixture of 5% (v/v) of each of the above-mentioned sera. Two questions needed to be answered. The first question was pertinent to the serum as a growth supplement and how it affected to total number of cells grown and harvested on a per mL basis. The second question related to the quality of the cells and the proportions that were found freely floating in the culture medium or anchored to the culture plate after 27 hours of growth. The ability of the cells to remain anchored throughout the course of experiments directly reflects the potential for accurate measurement of the results of the experiments. For example, if cells are washed out during the end-point detection portion of an experiment due to poor anchoring, their fate is not known regarding the parameters beign tested.

With respect to the total number of cells grown in each medium, the results were as follows: 5% (v/v) fetal + 5% (v/v) calf sera > 10% (v/v) fetal serum > 10% (v/v) calf serum; (*i.e.* 100% > 94.5% > 89.5%) However, in terms of anchorage to the plate and prevention of cell loss during washing and passaging, the results were as follows: 10% (v/v) calf serum > 10% (v/v) fetal serum > 5% (v/v) fetal + 5% (v/v) calf sera; (*i.e.* 94.5% > 93.9% > 91.7% cells retained).

2.1.2.2 METHODS AND MATERIALS

The materials used in the course of all experiments are listed in Section 2.2.

The medium to test this was prepared as follows: 900 mL of fresh Alpha Minimal Essential Medium was prepared, titrated to pH 7.10 using 5.0 N NaOH and sterilized by filtration through a filter kit with a 0.2 μm nylon filter. The fetal and calf sera were removed from freezing and thawed in a 37°C water bath. Each flask received a total volume of 10 mL thus, for three flasks, roughly 30 mL of medium was used. 8.0 mL of medium was delivered into each of three sterile 25 cm^2 culture flasks for mixing with serum. To the first was added 1.0 mL of fetal serum, iron enriched and supplemented to give a medium with a 10% (v/v) serum concentration. Similarly, to the second flask 1.0 mL of iron supplemented and enriched calf serum was added. To the third flask of medium was added 0.5 mL of each of the sera, giving a 10% (v/v) total serum concentration comprised of a mixture of 5% (v/v) calf + 5% (v/v) fetal sera. Each of the flasks were gently swirled to ensure proper mixing of the serum within the medium.

Cells were seeded into the three 25 cm^2 canted T-flasks by delivering 1.0 mL of a cell suspension containing 1.7 X 10^6 cells mL^{-1}. The flasks were again swirled gently to ensure homogeneous distribution of cells throughout the flask. In a total volume of 10 mL, the final cell count was 1.7 X 10^5 cells mL^{-1} in each flask. The cells were allowed to grow for an arbitrary 27 hours and were then harvested. The harvesting consisted of pipetting out the 10 mL of culture medium and delivering the contents of each flask to numbered sterile centrifuge tubes, corresponding to the flask number. Cells in the flasks

were washed with Dulbecco's phosphate buffered saline (DPBS) and then trypsinized using 0.25% trypsin. The contents of each flask was quenched with a solution of αMEM + 10% (v/v) calf serum. This cell suspension was delivered to numbered sterile 15 mL centrifuge tubes corresponding to the flask number. All of the tubes were centrifuged at 2,000 x *g* for 5 minutes. All of the supernatant was pipetted out after centrifugation and each pellet was resuspended in exactly 10 mL of medium. Cells from each flasks were thoroughly mixed and counted in triplicate on a haemocytometer.

2.1.2.3 RESULTS

Cell count of the cells grown in 10% (v/v) calf serum, was 436,000 cells mL^{-1}. The cell count of those grown in 10% (v/v) fetal serum was 460,000 mL^{-1}. Finally the count for those grown in 5% calf (v/v) + 5% (v/v) fetal sera was 487,000. The greatest growth (set at 100%) was seen in the 50/50 serum mixture, where the calf serum showed only 89.5% of the maximum, and the fetal serum showed 94.5%.

Distribution within the culture flask, *i.e.* the number of cells floating in the medium and those anchored to the culture plate was also considered. Comparison of the distribution of the cells in the flasks, showed that of cells grown in the 50/50 serum mixture 30,000 of the 487,000 cells per mL (6.2%) were free-floating. In the case of the fetal serum-grown cells 38,000 of the total 460,000 cells per mL (8.3%) were free-floating, while for the cells grown in calf serum, only 24,000 of the 436,000 (5.5%) were free-floating. This represents prevention of cell loss or retention of cells 93.9%, 92.7%

and 94.5% respectively. These results are found in Table 2.1.2.3.1, below.

Table 2.1.2.3.1

Percentage of Cells Recovered Based on Serum Type Added to Alpha Minimal Essential Medium as a Growth Supplement

Serum Type Added To αMEM	Number Of Cells In Supernatant	Number Of Cells In Monolayer	Total Cells Counted	Percent Recovered For Use
10 % Calf	24,000	412,000	436,000	94.5
10 % Fetal	38,000	422,000	460,000	91.7
5% Calf + 5% Fetal	30,000	457,000	487,000	93.8

2.1.2.4 DISCUSSION

The content and type of protein found in the two types of sera will affect the growth and anchoring ability of the cells. Fetal serum is one of the most effective supplements for cell growth due to its high content of embryonic growth factors (Butler, 1981). The use of calf serum affords the medium a greater concentration of fibronectin (DeVonne and Mouray, 1978), which is an important component in the anchoring process.

The values presented in the table below are not highly significant in themselves. However, one may wish to consider future experiments, where drug-treated cells may be lost after washing out of the medium due to insufficient anchoring ability. This loss of

cells coupled with other types of experimental error will likely result in highly inaccurate and questionable data. If one desires the ability of the cells to remain attached to the culture flask, the use of calf serum enhances this experimental requirement.

If anchoring ability of the cells is not a primary concern, it is worth noting that the cost of fetal serum is comparatively high while it contributed relatively little to the growth of the cells in this case. For this reason the serum of choice for the experiments to be conducted was calf serum.

2.1.3 Expression of Cell Growth

The growth of cells is a mathematically predictable process, which can be tailored to suit the needs of the investigator to a reasonable degree. It is not only a tool to control the experimental conditions, but it is a measure by which one is able to detect the subtle differences in the conditions of the cells being grown subsequent to an experiment or during the course of normal propagation of cells.

In order to better predict the time frame within which one expected the passing from the growth phase to the decline phase, while optimizing the amount of cells generated from a passage, the following equation was used. It allowed the investigator to predict either the time of generation of a certain number of cells from a known number seeded at a given time, or to determine the number of cells to be seeded in order to generate a certain number on a desired day.

$$N = N_0 \cdot e^{k\tau}$$

where N_o is the initial number of cells, N is the final number of cells, κ is the growth constant and τ, represents time.

2.1.4 Counting The Cells

Cell counting using the Hassuer Cell Counting Chamber, most often referred to simply as a haemocytometer, was used to determine the number of living cells that were trypsinized out of a cell culture flask. This was necessary as a matter of routine in order to correctly dilute and seed cells for propagation and for cytotoxicity experiments.

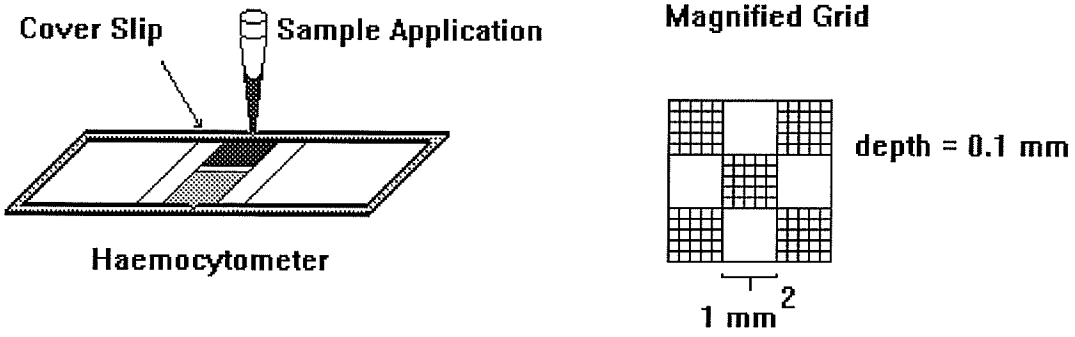


Figure 2.1.4.1 The Haemocytometer

(The above figure was redrawn from *Mammalian Cell Biotechnology: A Practical Approach*, Edited by Dr. Michael Butler, Oxford University Press, 1981).

It provided information not only with respect to how many cells were present, but by staining using a viability stain, trypan blue, the cell counter allowed for the differentiation between living and dead cells. The former appear clear under the microscope since they exclude dye, where the latter take up dye and appear dark blue.

The method for cell counting is as follows: Cells which are already in suspension are aseptically removed from a centrifuge tube or any other container. Usually a volume of 100 to 200 µL of cell suspension is sufficient. This volume is delivered to a 1.5 mL microcentrifuge tube. An equal volume of 0.6 % (w/v) trypan blue dye containing 1 mM EDTA is added to the microfuge tube and the mixture is triturated to ensure homogeneity. Once properly mixed, the cover slip is placed over the haemocytometer and approximately 10 µL volumes are delivered into a trough and the volume flows under the cover slip. The haemocytometer has nine large squares, each of which has a volume of 0.1 mm^3. This is equivalent to 0.01 µL. The cells are counted in five of the squares, usually the centre one and the four on the diagonal corners from the centre square. The number of cells found in the suspension is calculated using the following equation, where n equals the number of cells counted and the DF, or dilution factor is the dilution of the cells by the volume of dye added. DF was usually 2 since the dilution with dye was 1:1.

Number of Cells in Suspension per mL = $DF \cdot n \cdot 10^4/5$. (Figure 2.1.4).

2.1.5 *Determination of the Optimal Assay Time For Cytotoxicity Experiments With CHO-AA8 Cells*

2.1.5.1 INTRODUCTION

The deliberation over the correct or representative time frame for which to conduct a cytotoxicity experiment must reflect the ability of the drug to affect the cell within reasonable concentrations. The time frame must also allow for a reasonable number of cells to be seeded that will not compromise the undrugged controls by overgrowth. At the same time the duration of the experiment must allow for a detectable endpoint from among those populations severely damaged by cytotoxic drug. The selected time frame must also be able to function reasonably within the inherent problem of random errors in cell seeding process and exponential growth which tends to multiply these errors.

Experiments conducted by some investigators were often conducted for time frames as short as 1 hour (Dusre *et al.*, 1990; Alegria *et al.*, 1989), while others allowed the drug incubation to be 18 hours in duration (Wilson *et al.*, 1989). In order for drugs such as ICRF-187 to affect the cell, they must hydrolyze after passage into the cell. Also, a growth time far enough from the initial seeding is important to allow for the cells to move from lag phase to log phase and this according to this investigator's work, requires 12 hours in the case of the CHO-AA8 cells. Effects manifested in lag-phase cells are not representative of the metabolic potential of the cells. Thus, selection of the time frame must take into consideration the stability of the drug in certain medium conditions and the mechanism of the drug effect where it is understood.

2.1.5.2 METHODS AND MATERIALS

Three microtitre plates were seeded with 1000 cells per well. The volume of the cell suspension was 150 μL. After allowing the cells to anchor for 24 hours, ICRF-187 was prepared in a stock solution of 0.1 mg mL^{-1} (370 μM) in αMEM and serially diluted to give solutions of 37, and 3.7 μM. Final drug concentration in the wells was 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 μM. Drug was delivered using an Eppendorff pipette and all volumes were delivered to reflect proper drug concentration in a total final well volume of 250 μL. Drugging was done by delivery of a calculated volume of a drug solution of appropriate concentration or dilution to a well. Hence, volumes in the wells differed among rows in the plate and so, well volumes were adjusted to 250 μL after drugging by addition of appropriate volume of αMEM. The plates were numbered and each was allowed to incubate for different times in darkness at 37°C and in 95% air and 5% CO_2 (v/v). The first plate was allowed to grow in drug for 24 hours, the second for 48 hours and the third for 72 hours after the initial 24 hours anchoring. After each respective incubation in drug, 25 μL of 2.5 mg mL^{-1} MTT was added to each well and the plate was allowed to incubate for 4 hours. After development of the formazan dye, the wells were aspirated out using a bevelled 18G1½ needle attached to an water aspirator and then 100 μL DMSO was added to solubilize the formazan crystals developed by the live cells.

A similar experiment was conducted to measure the time dependent toxicity of doxorubicin. The doxorubicin was prepared in a 10 mM stock solution of 150 mM NaCl

and diluted 1:10 as required for experiments in sterile 150 mM NaCl solution. Solutions of 10, 100, and 1000 μM doxorubicin were used to deliver the final 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 μM drug concentrations in the wells. Volumes were made up to a final total of 250 μL with sterile 150 mM NaCl. In all other respects the experiment was conducted as the above ICRF-187 experiment.

2.1.5.3 RESULTS

Examination of the plates during the course of the experiments showed that by the third day the cells in the control wells were approaching 90% confluence. Though this gave strong absorbancies, the possibility of contact inhibition and cessation of logarithmic growth was a significant risk, since this would give erroneous results of the drugged wells with respect to the controls. Effects of doxorubicin after three days showed a very strong effect after 72 hours. Drugging at 0.1 μM for 72 hours killed 75% of the cells (IC_{50} = 0.18 ± 0.04 μM; data not shown). The proportion of kill was seen in 72 hours with 0.1 μM ICRF-187 was only 2-3%. Examination of the 24 hour drugging experiment showed that there was insufficient growth of the cells to give a strong absorbance. Most importantly, it did not afford sufficient sensitivity to distinguish between the subtle degrees of cell kill resulting from the small incremental changes in drug concentration from one set of wells to another. Absorbancies of the control wells for after-drugging growth times of 24, 48, and 72 hours resulted in absorbancies of 0.121, 0.312, and 0.926 at λ = 490 minus λ = 650 nm, respectively in the doxorubicin experiment. In the ICRF-

187 experiment, the absorbancies read at $\lambda = 490$ minus $\lambda = 650$ nm for the 24, 48, and 72 hours drug incubations were and 0.126, 0.382, and 0.757, respectively. The lowest absorbancies in both sets of experiments, were approximately 0.050 to 0.080 and so the differences between 0.080 and 0.126 *vs.* 0.321 are appreciable in terms of sensitivity and distinguishability.

2.1.5.4 DISCUSSION

The comparison of the 24, 48, and 72 hour drug incubation demonstrated that 24 hour incubation did not allow sufficient time for the growth of enough cells to distinguish between the subtle changes in cell death inflicted by the test drug. The 72 hour incubation resulted in extreme degrees of cytotoxicity, such that even in minute drug concentrations of the drug resulted in almost total kill. One serious and valid criticism of allowing a greater time for the cells to grow after drugging is that the small random errors inherent in seeding these experiments are exacerbated as cells grow at an exponential rate. Since cells growing longer will perpetuate this problem, longer growth times result in exponentially greater errors from the initial seeding number. The drugging time was selected as 48 hours and the experimental errors incurred by increased growth time was remedied by the use of six wells for each drug concentration measured. Thus, the power of statistics would weigh in favour of the statistically more valid number.

2.1.6 Selection Of The Optimal Endpoint Detection System for Measurement of Cytotoxicity

2.1.6.1 Selection of The Dye

Once the effects of a drug have taken place, an uncomplicated and reliable method by which to detect these effects was required. This method must be accurate, and must not be interfered with by the normal functions of the cell.

The selection of an appropriate method of measuring cell death inflicted by cytotoxic agents was made from a small number of end point detection systems, each of which was adaptable to automated ELISA (Enzyme-Linked Immuno-Sorbant Assay) or similar types of microtitre plate readers. The primary concerns in the selection of an assay system were the ease with which they could be used, low background noise and high sensitivity. Questions of interference with cellular enzymes or with the drugs being tested in the experiment were a concern as these kinds of interactions would give rise to questionable results. Finally, consideration had to be given to the target of the detection, *i.e.* live cells. The assays had to be able to discriminate between living cells and dead cells, and especially, freshly dead cells, which could result in artificially high survival rates.

The assays most strongly considered were sulforhodamine B (SRB) and MTT. The SRB assay is a protein stain while the MTT assay is a soluble tetrazolium salt which is cleaved enzymatically into an insoluble dark blue crystal by actively metabolizing cells. MTT, or (3-(4,5-dimethylythiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a biological

hydrogen acceptor (Alley *et al.*, 1988) which functions well as a detection system, but a number of parameters needed to be established before consistent and reliable results were obtained. (Vistica *et al.*, 1991).

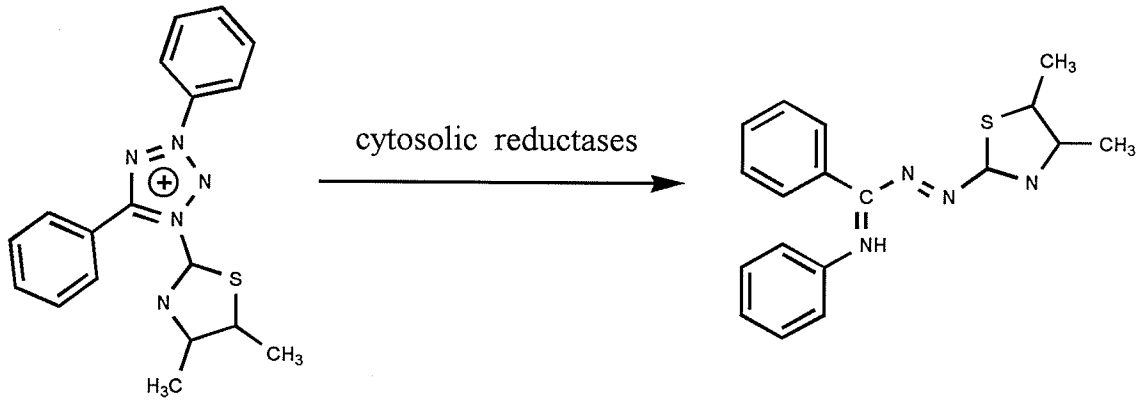
Comparisons were made to determine the useability and accuracy of these two assay systems. In the search for more practical and rapid automated methods for large scale tasks such as assays to screen hundreds of drugs for activity against the HIV virus, SRB is an advantageous method which offers accuracy as well as ease of use. (Rubinstein *et al.*, 1990). There were however, several unanswered questions regarding SRB that were solidly addressed by a large number of researches studying MTT. Many of those questions centred around whether or not the dye would interfere with the normal functions of cells or interact with the test drug, resulting in unreliable data.

Several relevant biological issues were pursued by investigators in an attempt to gain better understanding of the effects of MTT on the cell. The MTT salt shown in Figure 2.1.6.1 was studied by Mossman (1983), who claimed that the assay gave linear results from 200-50,000 cells per well. Preliminary work conducted by this author confirmed this data. (Data not shown.) This linearity range is important since the experiment seeded in this author's experiments were at 1000 cells/well and may easily have reach 50 x 10^3 cells per well in the total 72 hour duration of an experiment.

Studies by Marks *et al.*, (1992), showed that the rate of formazan crystal production differed for each different cell type, but noted that this is not a significant problem since most comparisons are made within a cell line, as was true for the research

presented by this author. Marks *et al.*, (1992), stated that drug resistant clones often metabolized MTT more slowly than the parental cells from which the resistant clones were derived. Beck *et al.*, (1979), suggested that this might be due to different growth rates between a parental line and the resistant line derived from it.

Figure 2.1.6.1 MTT Metabolism in the Cell



Yellow Tetrazolium Dye Blue Formazan Crystal

2.1.6.2 The Metabolism Of MTT

The question of MTT removal from drug-resistant cells by P-glycoprotein was addressed by Marks *et al.*, (1992), as well. It was thought that perhaps the increase in P-glycoprotein often found in resistant lines might remove MTT or change the cell membrane permeability to MTT resulting in the lower formazan production. Testing with an inhibitor of efflux activity, verapamil showed no change in the rate of formazan

production and so P-glycoprotein was not involved. This is important since the research presented in Chapter 5 of this thesis compares cytotoxicity of many drugs on CHO-AA8 cells (the parental line) with that of drugs on an ICRF-187-resistant line DZR3, derived from CHO-AA8.

Vistica *et al.*, (1991) states that contrary to early studies by Slater *et al.*, (1963) whose work was cited by numerous authors, including Mosmann, (1983), that MTT metabolism does not occur only in the mitochondria. MTT was hydrolyzed at the ubiquinone and cytochrome *b* and *c* sites of mitochondria (Slater *et al.*, 1963), but it was also widely used for histochemistry demonstration of specific non-mitochondrial enzymes (Pearse, 1972). MTT reduction was also often dependent on the maintenance of a threshold D-glucose level in the medium since low glucose resulted in decreases in NADH and NADPH and subsequent decreases in MTT reduction (Vistica *et al.*, 1991). The pH of the medium was not found to affect formazan production by cells. The culture age played a role in the rate of formazan reduction, where formazan production decreased with increase in culture age. All of the above mentioned parameters were held constant in the experiments presented in this thesis.

2.1.6.3 Solubility Of Formazan Crystals

Other questions were investigated by in the hope of improving upon the method first presented by Mosmann (1983). Mosmann used 100 µL of 0.04 M HCl in isopropyl alcohol to dissolve the formazan crystals. The problems cited above regarding the slow

production of formazan by slow metabolizing cells is compounded by stability and solubility problems of the formazan crystals. Often the difficulties in detection were not due to poor metabolism, but due to the inability to dissolve crystals which were shown microscopically to exist in copious amounts within the cells (Alley *et al.*, 1988). Studies done to improve upon solubilizing MTT compared solvents such as dimethyl sulfoxide (DMSO), mineral oil, and acid-isopropanol (Carmichael *et al.*, 1987) and hexane, dimethyl formamide (DMF), DMSO, propylene glycol, isopropanol, and acid-isopropanol (Alley *et al.*, 1988). Both groups found DMSO to be the best choice from among those solvents they tested in its compatibility with the polystyrene culture plates as well as solubility and stability and sensitivity.

MTT was selected as the endpoint detection assay for the research conducted in this project. It was used as described by Marks *et al.*, (1992), and DMSO was used as the solvent for the dye.

2.1.7 Biohazard Containment Certification

The safety requirements implemented in the general laboratory area were necessitated by the fact that mammalian cells were being manipulated. The choice as to which of the four levels of containment required was governed by the types of cells used or of potential use in the future. The cell line used for this project was a Chinese Hamster Ovary Cell, strain AA8. These cells were classified as Class I Biohazard by the ATCC. Class I cells require no special containment procedures, other than normal aseptic technique. However, cell lines potentially useful in the laboratory in the future included

PC-12, primary fetal mouse or rat myocyte cells, and MCF-7 (a human, breast adenocarcinoma). The MCF-7 line, because it is virally transformed, requires Class II Biohazard containment (*ATCC Quality Control Methods for Cell Lines, 1992*). Class II containment requires precautions such as the autoclaving of contaminated fluids, and of disposable and reusable cell culture materials. It further requires the use of a Class II laminar flow cabinet and confinement of the work to specified areas within the general laboratory. The safety requirements include annual testing of the flow cabinet to ensure correct air flow and proper filtration. Preparations of 1% sodium hypochlorite and 70% ethanol were in place in the cell culture lab areas at all times in the event of a contaminant spill. Eye wash stations and fire extinguishers were installed in the cell culture and general areas. Safety precautions were posted in several areas in the laboratory as were telephone numbers of all laboratory and safety personnel to be contacted in the event of a cell culture-related emergency. All persons working in the laboratory were required to conform to safety regulations implemented in the general laboratory area in order to comply with the Class II certification status.

Though these were standards to be adhered to in working with Class II biohazards, the standards were maintained in all work in this laboratory even though no Class II cells were in use. This included the washing and disposal of used pipette tips in a broken glass container, the disposal of used needles into a sharps container and the autoclaving of all disposable cell culture waste materials. The general certification applied for by and currently maintained by the laboratory was Class II.

2.2 Materials

2.2.1 Disposable Equipment

T-Flasks, (75 cm^2 and 25 cm^2, canted), Sterile Centrifuge Tubes (15 mL), Disposable pipettes [(1.0, 10.0, mL FALCON) and (25.0 mL, Baxter) sterile, individually wrapped)] were purchased from Corning (Corning, NY, USA) through Canlab Scientific Products. Falcon, Microtest III 96-well plates (flat bottom) as well as 24-well plates (flat-bottom) were purchased from Falcon through Canlab Scientific Products. 96-well plates (flat-bottom) and 10.0 mL pipettes (sterile, individually wrapped) were purchased from Sarstedt. Cryovials (1.8 mL), syringe filters (0.2 μm), Watman Laboratory Division (Clifton, NJ, USA) 4mm/PVDF/0.2μm/tube tip low protein binding filters for volumes < 2.0 mL, and disposable filter sterilization units (1L) were purchased from Nalgene through Canlab Scientific Products. 1.0 mL, 3.0 mL, 5.0 mL, and 10.0 mL sterile and individually wrapped syringes and 1.5 inch 18G needles were purchased through Canlab Scientific Products from Becton Dickenson. Sterile filter units with 1000 mL receivers and 500 mL reservoirs, 0.2 μm nylon filters (for tissue culture) were purchased from Nalgene through Canlab Scientific Products. Microcentrifuge tubes (1.5 mL), 1000 and 100 μL pipette tips, were purchased from CANLAB Scientific Products. Camco Quick Stain II Buffered Differential Wright-Giemsa Stain was purchased through Canlab Scientific Products from Baxter. Autoclavable borosilicate glass medium bottles, 250 and 500 mL were purchased from Corning through CANLAB Scientific Products. Drierite, anhydrous calcium sulfate was purchased from W.A. Hammond Drierite Company, (Xenia, OH, USA).

2.2.2 Chemicals

Alpha Minimal Essential Medium (αMEM) with Hank's salts and glutamine, without ribonucleosides, deoxyribonucleosides, sodium bicarbonate, calf serum (iron enriched and supplemented), Cell Culture Freezing Medium-DMSO, trypsin, (0.25% + 1mM EDTA), Antibiotic [10,000 units mL^{-1} Penicillin G (sodium salt) + 10,000 μg mL^{-1} Streptomycin sulfate] and sodium bicarbonate (Cell Culture Tested), were all purchased from Gibco BRL (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), Trypan Blue, Trizma Base Buffer, N-2-Hydroxyethylpiperizine-N'-2-ethanesulfonic acid (HEPES) Buffer (Cell Culture Tested), $4Na^{+}$• EDTA (Cell Culture Tested), Sodium Pyruvate (Cell Culture Tested), NADH and Dulbecco's Phoshate Buffered Saline (DPBS) (without $MgCl_2$ or $CaCl_2$) were all purchased from SIGMA Chemical Company (St. Louis MO, USA). Dimethyl Sulfoxide (spectral grade), and standard NaOH and HCl solutions were purchased from Mallinckrodt (Paris, KY, USA). Carbon Dioxide (standard grade) was purchased from Welders' Supply (Winnipeg, Manitoba, Canada). Chinese Hamster Ovary Cells (AA8) were purchased from American Type Cell Culture (ATCC), (Rockville, MD, USA).

2.2.3 Drugs

Doxorubicin, daunorubicin, epirubicin, idarubicin, ICRF-186 (levroxazane) ICRF-187 (dexroxazane) and ADR-925 were gifts from Adria-SP Inc. (Columbus, OH, USA). Mitoxantrone was a gift from Lederle Laboratories Division, American Cyanamide Company, (Rearl River, OH, USA) ICRF-154, ICRF-161, ICRF-192, ICRF-193, ICRF-

197, AL-1567 and ADR-559 were gifts from Dr. A.M. Creighton,. BLPD2GA, BLPDB2, BLPDC4, and BLPDE1 were synthesized by Mr. Bin Ling in the Faculty of Pharmacy, University of Manitoba. Verapamil, vinblastine, etoposide, cyclophosphamide, amethopterin, cisplatin, and 5-fluorouracil were purchased from Sigma Chemical Company (St. Louis MO, USA). Hydroxyurea was a gift from Dr. Grant McClarty, Medical Microbiology, Faculty of Medicine, University of Manitoba.

2.2.4 Laboratory Equipment

Cells were counted on a Hausser Scientific Partnership (Horsham, PA, USA) counting chamber using a Nikon TMS inverted microscope. Cells were photographed using a Nikon FE body loaded with Kodacolor 200 and mounted on a Nikon Labophot-2 Microscope. Photographic prints were made on Kodak color print paper. All High Performance Liquid Chromatography was performed using a Varian Star 9010 Solvent Delivery System and a Varian Star 9050 UV-Visible Detector, both run by Varian Star software. MTT Assays were read using a Thermomax Plate (Mandel Scientific) reader or on an EAR 400 Plate reader. All Data was processed using SigmaPlot 5.0 and SigmaStat software. Pipetting was done using a Gilson 8 x 200 µL multichannel pipettor (for cytotoxicity assays, MTT and DMSO delivery) and Eppendorff 0.5-10.0 µL, 2-10 µL, 20-200 µL, 50-250 µL, and 100-1000 µL pipettes for cytotoxicity assays and other pipetting. Cells were grown in a National Appliance Company Model 3331 Incubator (Portland, OR, USA) with water-jacketed temperature control and dual CO_2 controls.

Cell manipulations were performed in a Envireco (Albuquerque, NM, USA) (laminar flow hood) with a HEPA filter. Double distilled water was prepared using a Corning (Corning, NY, USA) Mega-Pure 1 L glass still.

2.3 Preparation Of Commonly Used Solutions

The preparation of commonly used solutions is described in the following paragraphs since their use was widespread. The majority of these solutions were used on a daily basis and a single general all encompassing description is recommended over redundant description during the course of each experimental procedure.

2.3.1 Alpha Minimal Essential Medium

The cell culture medium used to grow Chinese Hamster Ovary AA8 cells was αMEM (with and Hank's Salts and glutamine, without ribonucleosides without deoxyribonucleosides, without sodium bicarbonate). It is purchased in packets of premeasured powder which will make 1 L. In its powdered form, it was stored in the refrigerator at 4°C. Its preparation was as follows: Note that the preparation of 1 L of medium includes 10% (v/v) serum supplement, and so only 900 mL of actual medium were prepared prior to the addition of serum. 800 mL of glass double distilled water were delivered to a 2 L beaker. The beaker was placed on a magnetic stirrer with a magnet and it was set to stir the water fairly quickly. The foil package containing the medium was carefully cut with scissors and the contents were emptied into the beaker of glass double distilled water. The powder which remained in the packet was washed out with double

distilled water from a squeeze bottle and poured into the medium solution. 2.2 grams of sodium bicarbonate were added to the stirring solution, as per manufacturer's instructions. The sodium bicarbonate acts as a buffer in conjunction with the 5% carbon dioxide flowing in the growth chamber. In order to better control the pH of the medium during experiments and propagation of cells, 20 mmol of HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer were added to the solution per litre. (The molecular weight of HEPES is 238 grams mol^{-1} and so 4.76 grams were added per litre of medium.) It must be noted that buffers such as HEPES must be added with care since optimal osmolality of standard growth medium is approximately 300 mOsm kg^{-1} and this is an optimal osmolality in which most cells lines will grow. In most cases a change of 5-10% or up to 25 mM HEPES in the osmolality is tolerated by the cells while maintaining the pH between 6.9 and 7.4 (Butler, M., 1981). Addition of 20 mM HEPES did not exceed a 10% change in the medium osmolality and cell growth was not affected thus, no other changes were made in the preparation of the medium.

After all of the powders were dissolved the volume was made up to 900 mL as accurately as possible. The solution was titrated to a pH of 7.10 using standardized 5.0 M NaOH. The pH of the glass double distilled water was measured as 4.86 and thus, effort was made to make up the fluid volume to 900 mL as accurately as possible with the water prior to titration since addition of the water after titration to the desired pH would be undermined by the addition of the acidic water. The pH of the water is due to absorption of atmospheric CO_2 , which results in an increase in the acidity of the water.

(Note that titration of the medium to a pH of 7.10 prior to filtration resulted in a post-filtration pH range of approximately 7.43-7.40 and a pH of 7.38 after addition of calf serum to 10% (v/v). Measurement of the medium pH after incubation in 95% air and 5% CO_2 for 24 and 48 hours gave pH readings of 7.43 and 7.30, respectively.) The solution was sterilized using a disposable vacuum filter kit with a 0.2 μm nylon filter, a 500 mL reservoir and a 1000 mL receiver. A nylon filter was selected in order to minimize binding of proteins during filtration. Vacuum was generated by a water aspirator. (Note that the solution was not autoclaved, since it is unable to withstand the high temperatures).

Once the filtration was complete the entire filter kit was taken to the laminar flow hood and the receiver was removed and discarded. Antibiotic [10,000 units mL^{-1} penicillin G (sodium salt) plus 10,000 μg mL^{-1} streptomycin sulphate] was added to control bacterial contamination in the concentration of 100 units mL^{-1} penicillin G plus 100 μg mL^{-1} streptomycin sulphate per litre of medium. Thus, to each 1000 mL of medium, 10 mL of reconstituted antibiotic was added. Reconstitution of the lyophilized antibiotic was done by adding 20 mL of glass double distilled water to the antibiotic bottle and swirling until all of the powder was dissolved. The medium was swirled gently to distribute the antibiotic. The function of the individual antibiotics were as follows: Penicillin at concentrations of 100 IU mL^{-1} was added to inhibit the growth of Gram-positive bacteria, while streptomycin in concentrations of 50-100 μg mL^{-1} was added to inhibit the growth of Gram-negative bacteria. Antifungal agents were not used in this preparation. The

medium was carefully poured over into two sterile 500 mL bottles in 450 mL volumes.

At this point 50 mL of freshly thawed iron enriched and supplemented calf serum was added to one of the 500 mL bottles. This bottle was now ready for use. Serum was not added to the second bottle until the first bottle was consumed in order to prevent the wasting of serum should the medium not be used within 20-30 days, which is the shelf life of reconstituted medium containing glutamine (Butler., 1981).

2.3.2 Dulbecco's Phosphate Buffered Saline (DPBS)

DPBS was purchased in preweighed packages which make 1 L when dissolved. The crystalline preparation was stored in the -20°C freezer until needed. When required, the bottle containing powder for 1 L was removed from the freezer and allowed to warm in a desiccator. When warm, the seals of the bottle were removed and the contents were delivered into a 1000 mL volumetric flask. Approximately 900 mL of glass double distilled water was added to the flask and the contents remaining in the bottle were washed out into the flask of solution using glass double distilled water from a squeeze bottle. A magnet was put into the flask and the solution was stirred magnetically to ensure homogeneity. Once the salt was dissolved the magnet was removed and the volume of the solution was made up to 1000 mL. The solution was sterilized using a disposable vacuum filter kit with a 0.2 μm nylon filter, a 500 mL reservoir and a 1000 mL receiver. Vacuum was generated by a water aspirator. (Note that the solution was not autoclaved, since it is unable to withstand sterilization temperatures). Once the filtration was complete the entire filter kit was taken to the laminar flow hood and the receiver was removed and

replaced with a sterile bottle cap. The solution was stored at 4°C.

Measurement of pH of the DPBS solution prior to filtration was 7.52 and after filtration pH was measured at 7.50. This was satisfactory.

2.3.3 Sterile Double Distilled Water

Sterile water was often required for dilution of drugs, reconstitution of lyophilized antibiotics and for making up of water volume lost due to autoclaving of solutions.

Its preparation was as follows: Glass double distilled water was delivered in volumes of approximately 100 mL to clean autoclaveable borosilicate glass bottles. These bottles were capped, but not tightly, and autoclaved at 250°C for 20 minutes. Once the autoclaving cycle was complete the bottles were removed from the autoclave, allowed to cool in a clean dust-free environment and then the caps were tightened. The bottles were stored at room temperature. Note: The caps were tightened only after the bottles had cooled to room temperature to prevent the generation of negative pressure within the cooling bottle, that when opened, may pull dust or bacteria into the sterile bottles and contaminate them.

2.3.4 Sterile 150 mM NaCl Solution

Sterile 150 mM NaCl was required for the dilution of anthracyclines, MTT solution, as well as for the making up of microtitre plate well volume with physiologically osmolar liquid that is neutral in its effect during cytotoxicity assays.

Its preparation was as follows: The required volume was made by measuring 90% of the required volume of glass double distilled water into a beaker of appropriate size.

Usually five bottles of approximately 100 mL each were made at a given time, thus a 600 mL beaker was used. The appropriate weight of NaCl was weighed out and dissolved in the water. The solution was transferred to a volumetric flask of appropriate volume (*i.e.* 500 mL) and the volume was made up with double distilled water. The solution was stirred with a magnetic stirrer and then delivered in approximately 100 mL volumes to clean, labelled, autoclavable borosilicate glass bottles. The caps were screwed on loosely and the bottles were weighed. The weights of the bottles were recorded on the bottles and the bottles were autoclaved for 20 minutes at 250°C. After the autoclave cycle was completed, the bottles of NaCl solution were removed from the autoclave and allowed to cool in a clean place. The bottles are reweighed and the weights recorded on the bottles. The difference in the masses of the bottles before and after sterilizing was due to evaporation of water during autoclaving. Thus, the bottles are then taken into the sterile environment of the laminar flow hood an the appropriate volume (1 mL per gram of water lost) was added to each bottle. The bottle caps were tightened and the NaCl solutions were stored at room temperature.

2.3.5 Preparation And Use Of MTT

MTT solution was prepared by weighing out the appropriate mass of MTT crystals to be dissolved 2.5 mg mL^{-1} in 150 mM NaCl solution or DPBS to give the desired volume. For example, if 25 mL of MTT solution were required at a concentration of 2.5 mg mL^{-1}, was weighed out 2.5 mg mL^{-1} X 25 mL = 625 mg or 0.625 grams of MTT. 25 mL of 150 mM NaCl solution or Dulbecco's PBS was delivered, the solution was

vortexed to dissolve all of the crystal. It was stored in the refrigerator at 4°C in a sterile borosilicate glass bottle wrapped in aluminum foil to prevent photodegradation of the light sensitive solution.

The method used for endpoint detection was a modification of Mosmann's method described by Marks *et al*, 1992. The MTT was delivered 10 µL per 100 µL of well medium. Thus, since the experiments were conducted (with few exceptions) using a final medium volume of 200 µL $well^{-1}$, 20 µL of MTT solution was added to each well. The wells were allowed to incubate in the dark at 37°C and in 95% air and 5% CO_2 (v/v) for four hours.

After development of the crystals all of the medium was pipetted out and 100 µL DMSO was added to each well to dissolve the crystal. Special care was taken in removing the medium following the incubation with MTT. Though numerous authors suggest either centrifuging of the entire plate to precipitate crystals or alternatively, leaving behind 10 to 20 µL of medium (Alley *at al.*, 1988), the most suitable approach for this investigator was to carefully remove all of the medium without disturbing the crystals or the cells containing them. The best method for this was to aspirate the wells using a water aspirator coupled with a 1 mL syringe and a 1.5 inch 18 gauge needle. The plate was tilted approximately 45-60 degrees and the bevelled edge of the needle was slid down the side of the well so only the very tip of the needle touched the bottom of the well. None of the cells or the formazan was disturbed. Though it may be argued that there might have been a significant loss of formazan crystal, it is also true that only the formazan from

positively live cells was dissolved by the DMSO. In any case, what was washed out did not create significant error since the standard deviations after some practice, were often as low as 2-3% in many cases and were commonly 5-10 % and very rarely exceeded 20%.

The absorbances were read on a Thermomax at 490 nm with a reference beam at 650 nm. Best results were obtained if the plate with DMSO was allowed to sit at room temperature in the dark for a minimum of three hours prior to reading. This resulted in better solubilizing of the formazan and thorough distribution of the dye throughout the well. The same was rarely accomplished using the shaker function of the plate reader even when shaking was repeated 3-5 times.

2.3.6 Trypan Blue Dye

Trypan Blue dye was required for cell counting. It allowed one to distinguish live cells from dead ones in that the live ones did not allow the dark blue dye into the cell, while the dead ones were stained by the dye and appeared as dark blue on the counting chamber. A pure stain without debris was required and so the following protocol by Seglin (1976) was used.

Preparation was as follows: Trypan Blue dye was weighed out in order to prepare 100 mL of 0.6% Trypan Blue dye (w/v); 0.6 g of the dye was weighed out and added to 100 mL of double distilled water. EDTA was weighed out to give a 1 mM solution in the 100 mL.

The solution was vortexed to dissolve as much of the dye as possible. The dye was then delivered to centrifuge tubes and centrifuged at 10,000 rpm for 60 minutes in order to precipitate all particulate matter. After the centrifugation the supernatants were carefully collected and filtered using 0.2 μm syringe filters. The dye was delivered in 10 mL aliquots to 10 centrifuge tubes, and frozen at -20°C. The dye was thawed one tube at a time and used.

2.3.7 Preparation of Commonly Used Drugs

ICRF-187 and doxorubicin were the most commonly used drugs in the course of this investigator's research. Consequently they were prepared routinely. This is especially true for ICRF-187 which was prepared fresh daily, since stable storage in a non-acidified form was not suitable. Described below are the detailed procedures for the preparation of these drugs. The methods described were used throughout the course of this investigator's work and will not be described elsewhere unless changes were made.

2.3.7.1 Preparation of ICRF-187 in α-MEM

Preparation of ICRF-187 was done on the day of a drugging as close to the drugging time as possible, since its half-time of hydrolysis at neutral pH was approximately 9.3 hours (Buss, J.L. and Hasinoff, B.B., 1993). The form most readily able to traverse cell membranes is the parent, or ICRF-187 (Figure 3.1.3.1.2) from of the drug, while the metal chelating **B**, **C**, and ADR-925 forms were unable to pass through the membranes of the cells. Thus, to retain the parental form and prevent its premature base-mediated hydrolysis to **B**, **C**, and ADR-925 forms at the pH of the medium, the drug was

prepared no more than 20 minutes prior to its use. If it was not used immediately after it was dissolved and filter-sterilized, it was stored on ice for the short time until it was used.

The recrystallized ICRF-187 was stored in a brown bottle at -20°C in a sealed plastic container with Drierite desiccating chips. When the drug was to be made up, it was removed from the freezing container and allowed to warm to room temperature in a desiccator containing Drierite chips. The required amount of drug was weighed out and delivered to a disposable 10 mL borosilicate glass test tube. The concentrations most often made up were 1 mg mL^{-1} (3700 μM), though more convenient concentrations were also prepared; 10 mM (2.68 mg mL^{-1}), and 20 mM (5.36 mg mL^{-1}). The solvent for ICRF-187 was always α-MEM, which was removed from refrigeration and warmed in a 37°C water bath prior to use. Dissolving the drug at the 1 mg mL^{-1} concentration was not difficult however, the preparation of 10 and 20 mM solutions required 20-30 minutes of sonication.

After the drug was completely dissolved, the solution was filter sterilized to prevent contamination during the experiment. This was done using an appropriate size of disposable syringe and a 0.2 μm filter syringe. In the case of small volumes, such as 1 or 2 mL, the drug solution was drawn up from the test tube using the syringe and a 1.5 inch 18 gauge disposable, sterile needle. After drawing up of the drug, the needle was removed from the syringe barrel and replaced with the filter and filtered. The filtrate was delivered to a sterile disposable centrifuge tube. In the case of large volumes of drug

solution, a sterile 10 mL syringe was used. The plunger was removed and the filter was secured to the locking end. The filter spout was placed in a sterile centrifuge tube, the drug solution was carefully poured into the open end of the syringe and the plunger was re-inserted to force out the solution. The drug was then ready for dilution and delivery.

2.3.7.2 Preparation of Doxorubicin Solution

Preparation of the Drug Solution

A 10 mM stock solution of doxorubicin was prepared in double glass distilled water. The powdered drug was stored at 4°C and was removed from the refrigerator and allowed to reach room temperature in a desiccating vessel prior to weighing. The drug was prepared by weighing out ~ 5.8 mg mL^{-1} of drug to be prepared. (The molar mass of doxorubicin·HCl = 579.99 g $mole^{-1}$). Since doxorubicin is photosensitive, the powder was delivered to a borosilicate glass test tube wrapped in aluminum foil to prevent photodegradation of the doxorubicin. The appropriate volume of glass double distilled water was delivered to the test tube and the drug was dissolved readily in the water, but without shaking in order to prevent coating the glass surfaces and losing drug to the glass.

Spectrophotometric Determination of Doxorubicin Concentration

Since the purity of the drug is not known it was analyzed spectrophotometrically in methanol using the molar absorbtivity (ε) of 12200 M^{-1} cm^{-1} at 480 nm (Burke *et al.*, 1987). Determination of the doxorubicin concentration required a stoppered quartz cuvette which was washed with 95% ethanol, air dried, and weighed with the stopper. HPLC grade methanol (MeOH) was added to the cuvette to about two thirds of the cell volume

using a pasteur pipette. The cuvette was stoppered, and any residual MeOH on the surface as allowed to evaporate off. The cuvette and MeOH were weighed and the volume of the MeOH was calculated from the known mass and density of the MeOH. A 5 μL volume of the drug was delivered to the cuvette which was stoppered and inverted once to mix the drug in the MeOH. The cuvette was placed into the spectrophotometer (blanked earlier with the same cuvette containing only MeOH) and the absorbance was read at 480 nm. The absorbance was recorded and the concentration of the drug in the MeOH was calculated from the molar extinction coefficient (ε) and the absorbance, A. The actual doxorubicin solution concentration was then calculated knowing the volume of MeOH in which the drug was diluted out for measurement.

Sample calculations:

Mass of dry stoppered cuvette + MeOH	14.290 g	
-Mass of dry, stoppered cuvette:	-13.436 g	
Mass of MeOH		0.864 g

Density of MeOH = 0.7914 g mL^{-1}.

$$0.7914 \text{ g}/ 1000 \text{ μL} = 0.864 \text{ g}/\chi \text{ μL} \qquad \chi = 1091.73 \text{ μL MeOH}$$

Absorbance of doxorubicin read in MeOH = 0.583 at 480 nm.

From Beer-Lambert Law: A= εcl, where ε is the molar extinction coefficient of doxorubicin in methanol, c is the concentration and l is the length of the path of light travelling through the cuvette. The ε = 12200 M^{-1} cm^{-1}, l = 1 cm, and A is the sample absorbance, 0.583.

$$0.583 = (12200 \text{ M}^{-1} \text{ cm}^{-1})(c)(1.0 \text{ cm}) \therefore c = 0.047787 \text{ mM in MeOH.}$$

The above concentration represents the concentration of the drug in the MeOH. The volume of the MeOH calculated above is used to determine the concentration of the stock doxorubicin solution prepared. The volume of the MeOH is added to the 5 μL drug

solution added to the MeOH to give the total dilution factor and the drug-in-MeOH concentration is multiplied by the dilution factor to give the stock drug concentration.

1091.73 μL MeOH + 5 μL doxorubicin = 1096.73 μL.
0.047787 mM x 1096.73 μL = 52.409605 mM (5 μL^{-1})

To find the concentration mL^{-1},

[52.409605 mM (5 μL^{-1})] x (1000 μL mL^{-1}/5 μL) = 10.48 mM

This method was also used to prepare daunorubicin and epirubicin where the molar absorbtivities were 12100 and 12200 M^{-1} cm^{-1} at 478 and 480 nm respectively (Burke *et al.*, 1987). No molar absorbtivity is known for idarubicin thus, it was prepared by weight.

2.3.8 Cryogenic Freezing of Cells And Their Recovery After Freezing

Cells are stored over long periods of time in liquid nitrogen. Freezing in this way allows for long term storage with survival of a large fraction of the cells. However, the cells must be prepared for the freezing. In order to prevent damage to the cells by the formation of ice crystals, the cells were suspended in a cryoprotective agent, usually a growth medium supplement supplimented with 5% DMSO or 10% glycerol. The former is usually preferred since its rate of penetration into the cell is greater. Care must be taken however, to minimize the cells' exposure to DMSO above freezing temperatures since this can damage the cells (Butler, M., 1981). The procedure is described below.

Freezing The Cells

Usually in freezing cells, enough must be grown that about 10 1.0 mL cryovials of cells containing 4-5 million cells per mL are frozen. The large number per mL is

required to ensure that enough cells survive the freezing to begin growing. CHO-AA8 are usually viable to 90% or more, however, it is a worthwhile precaution. Also in the event of a freezing mishap, it is better to have several extra vials rather than not enough. After the number of cells required for freezing had been grown, the cells were washed with DPBS, trypsinized with 0.25% trypsin, and quenched, the cells were centrifuged and then pooled in a 10 mL suspension. The cells were counted and then centrifuged again. After centrifugation, the cells were resuspended in cell culture freezing medium containing 5% DMSO in a volume that gave a cell concentration of 4-5 million cells per mL. The cells were delivered in 1.0 mL aliquots into prelabeled 1.8 mL cryovials and placed upright into a small glass beaker. This beaker was put into a styrofoam ice container and frozen over night at -60°C. The ideal freezing rate for cells is 1°C/minute. It is this particular freezing procedure which is critical to the cells' survival . The cells must freeze quickly enough to minimize the damage potentially caused by DMSO, but not too quickly, so that the cells are sheered by ice crystals. After freezing at -60°C, the cells were removed from the freezer, and the individual vials were placed into the storage canes and immersed in the liquid nitrogen tank.

Thawing The Cells After Freezing

The cells were removed from the cryogenic tank and the cap was loosened slightly to allow for escape of the rapidly expanding nitrogen gas. The vial was then immediately immersed in a 37°C water bath to rapidly thaw the cells. Note that slow thawing after cryogenic storage is detrimental to cell survival. The cells are pipetted out of the vial and

delivered into warm cell culture medium. The cells in medium is placed into the incubator to grow. The cells were passaged twice before they were used for experiments.

2.3.9 Maintaining The CO_2 Levels And Temperature Within The Growth Chamber

The incubator used for growing cells was maintained with a continual gas flow of 95 % air and 5% CO_2 (v/v) and its temperature was regulated at 36.5 ± 0.5 °C. The CO_2 in the growth chamber was supplied by pressurized CO_2 cylinder. The CO_2 flow was maintained at 5% (v/v) by regulating the flow in the growth chamber, which was set to 0.2 L/minute while air flow from a main air line was filtered and allowed to flow into the chamber at 4 L per minute. The level of CO_2 in the chamber was measured and verified as being 5% (v/v) by a Fierite CO_2 meter.

The temperature was set to 36.5 °C with the safety cut off set to 37°C. This allowed for a narrow and well maintained temperature range that was optimal for the cells. The temperature was confirmed by a thermometer which remains in the chamber at all times immersed in a small bottle of water. Humidity in the chamber was maintained by a tray situated on the bottom of the chamber which held approximately 1 L of water. The water was kept free of microbes and fungi by the addition of 1 gram of copper sulfate to the tray.

Chapter 3

SINGLE AGENT CYTOTOXICITY EXPERIMENTS

3.1 SINGLE AGENT CYTOTOXICITY EXPERIMENTS

3.1.1 INTRODUCTION

A series of single drug experiments was conducted on Chinese Hamster Ovary AA8 cells to measure and compare the cytotoxic effects of two structurally different groups of bisdioxopiperazines, the ICRF-Series and the BLDP-Series.

The toxicity of a number of these agents was also examined in light of lipophilicity data presented by Creighton *et al.*, (1979) to determine whether or not there is a significant relationship between membrane permeability and cytotoxicity. Topoisomerase II inhibition studies conducted by Yalowich *et al.*, (unpublished) were compared with IC_{50} values determined in this series of experiments to clarify whether or not the toxic effects mediated by some of these drugs were related to the inhibition of this DNA winding and unwinding enzyme.

3.1.1.1 *The Bisdioxopiperazines*

The bisdioxopiperazines that were tested for cytotoxicity against Chinese Hamster Ovary AA8 cells were structurally different. The first set of drugs tested was the ICRF series, synthesized by Creighton (Creighton *et al.*, 1979). These included a number of structurally similar antitumor agents known as ICRF-154, ICRF-159 (razoxane), ICRF-161, ICRF-192, ICRF-193, ICRF-197, as well as ADR-559. Of central importance to the cytotoxicity research conducted were ICRF-186 (levrazoxane), the (-)-(*R*) isomer of ICRF-159, and ICRF-187 (dexrazoxane, ADR-529), the (+)-(*S*) isomer of ICRF-159. The

intermediate metabolic hydrolysis products of ICRF-187, compounds **B** and **C**, as well as the final hydrolysis product of ICRF-187, ADR-925 were tested for their cytotoxic effects on CHO-AA8 cells.

The second group of bisdioxopiperazines tested for cytotoxicity against CHO-AA8 cells was the BLPD series, which consisted of four compounds, BLPDG2A, BLPDB2, BLPDC4, and BLPDE1.

3.1.2 METHODS AND MATERIALS

3.1.2.1 Seeding of Microtitre Plates With Cells

Cells were grown to the point of near confluence in a 25 cm^2 or 75 cm^2 canted T-flask at 37°C in an atmosphere of 5% CO_2 and 95% (v/v) air. They were washed with Dulbecco's PBS and trypsinized with 0.25% trypsin + 1 mM EDTA. After separation of the cells from the plate surface, as observed under the microscope, the trypsin was quenched with medium using a medium:trypsin ratio of 10:1. The cells were centrifuged at 2000 x *g* for 5 minutes. The supernatant was removed and the cells were resuspended in 10 mL of medium. The cells were counted on a haemocytometer six times, and the numbers were averaged. The cells were diluted in an appropriate volume of medium to give a cell count of 10,000 cells/ mL. Each well was seeded with 100 µL of cell suspension to give a cell count of 1000 cells/well. The volume of cell suspension prepared was in accordance with the number of plates to be seeded, each plate requiring approximately 10 mL of cell suspension. The cells were delivered to the microtitre wells

using an Eppendorff repeater pipet fitted with a 5 mL barrel with the repeater set to deliver volumes of 100 μL. Once seeded, the microtitre plates were placed into the incubator for 24 hours to allow the cells to anchor prior to the drugging.

3.1.2.2 Drug Preparation

Preparation of the drugs has been discussed generally in 2.3.7.1. Preparation of ICRF-154, ICRF-159, ICRF-161, ICRF-192, ICRF-197 and ADR-559 was the same for each of the drugs. The only difference in their preparations was the concentrations at which stock solutions were prepared. Stock preparations were a function of the drugs' solubilities in cell culture medium. While ADR-559, ICRF-159, ICRF-161, ICRF-186, ICRF-187, compounds **B**, **C**, and ADR-925 were easily prepared at concentrations of 1.0 mg mL^{-1} or greater, ICRF-192 and ICRF-154 were not soluble above 0.1 mg mL^{-1}. A typical drug preparation and dilution scheme for drugs easily dissolved in αMEM is illustrated in Figure 3.1.2.2.1.

Drugs soluble at 0.1 mg mL^{-1} or less were dissolved in cell culture medium using sonication for periods of at least 10 minutes. Once the stock solutions were prepared, they were filtered and diluted as described in 2.3.7.2. In dissolving the BLPD series drugs, 20 to 30 minutes of sonication were required to dissolve the drug at 0.1 mg mL^{-1}.

A different procedure was used to dissolve and deliver the sparingly soluble ICRF-193 and ICRF-197. Since their solubilities at 0.1 mg mL^{-1} were not complete in cell culture medium, DMSO

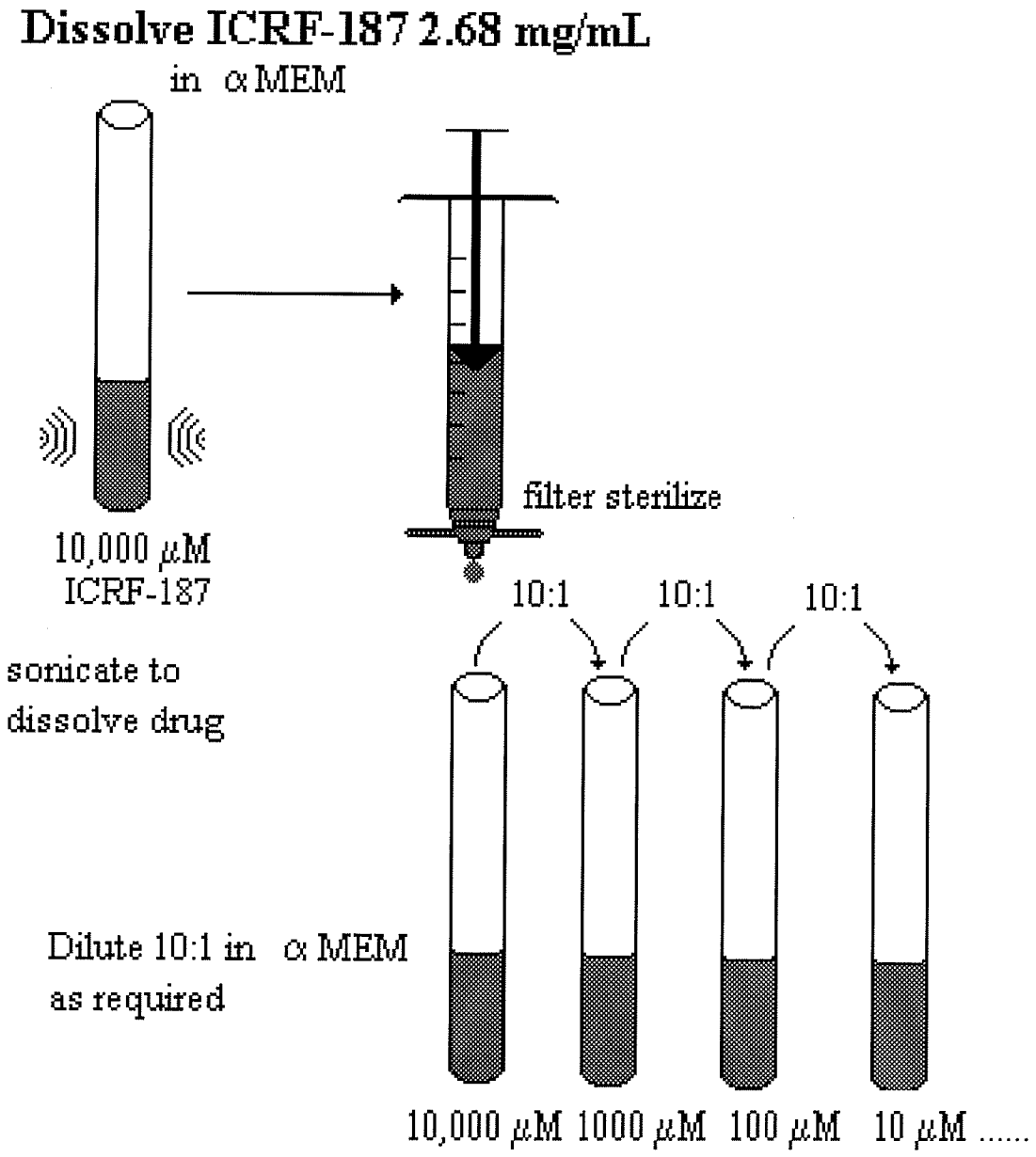


Figure 3.1.2.2.1 **Drug Dilution Scheme For αMEM Soluble Drug**

was used to dissolve the drug. Since DMSO is itself toxic, it was necessary to determine whether or not there was a safe volume which could be delivered to a well that would not compromise the integrity of the experiment. A preliminary experiment was conducted which involved delivering between 0.1 and 50% DMSO per well and measuring the degree of toxicity mediated by DMSO. MTT end-point detection was used to measure the cell kill. The experiment showed that from 0 to 0.9% (v/v), DMSO was not detected as being toxic to cells. (Figure 3.1.2.2.2)

The drug was dissolved in DMSO at 2.82 mg mL^{-1}. A sample of 0.8000 mg was weighed out and dissolved in 283.6 μL of pure spectral grade DMSO to give a solution of 10,000 μM ICRF-193. This solution was serially diluted 10:1 in DMSO to give a series of 100 μL volumes of 1000, 100, and 10 μM drug. Each of these was further diluted to give incremental concentrations of drug, which spanned from 2.0 to 10,000 μM. These drugs were now at their correct concentrations and were delivered in 1.0 μL volume per well containing a total final volume of 200 μL. After dilution in the 200 μL the range of drug concentrations in the experiment was from 0.01-50.0 μM. The rationale behind this method was two-fold. The DMSO was used as it was easily able to solubilize the drug. At such high concentrations, the volume delivered into each well was kept low. This was desirable since above 0.9% (v/v), DSMO manifests its own toxic effects. By delivering 1.0 μL in a total volume of 200 μL volume, the DMSO added per well was kept constant and at 0.5% (v/v). A preparation and dilution scheme for drugs dissolving in DMSO is illustrated in Figure 3.1.2.2.3. The actual drug delivery is described further below.

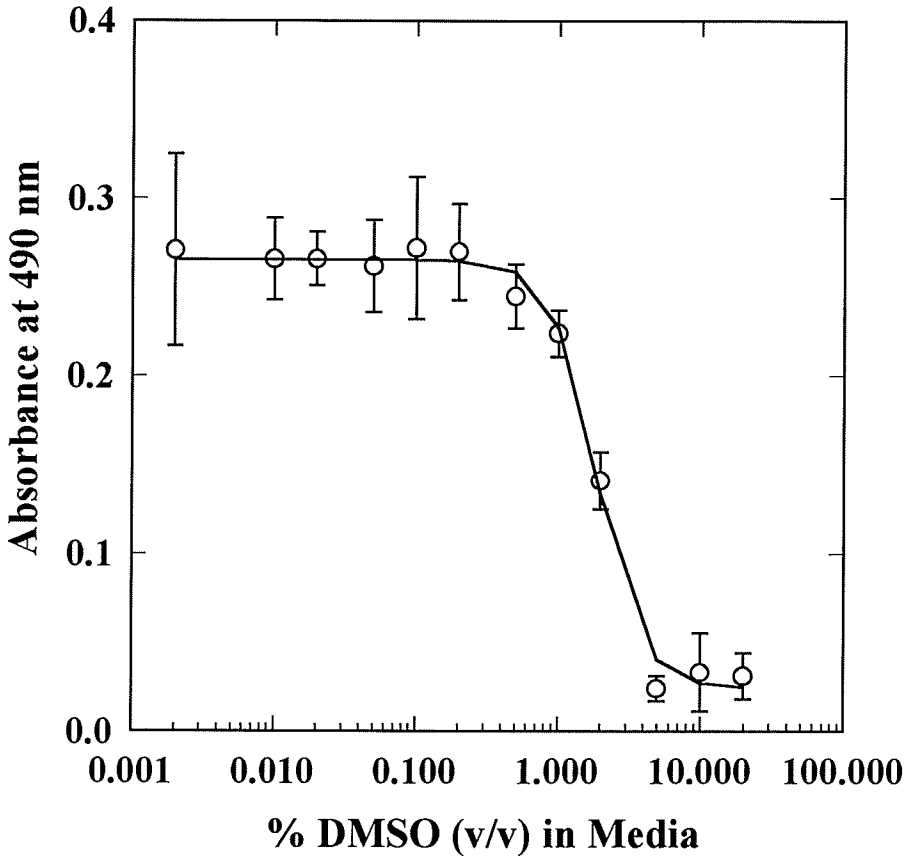


Figure 3.1.2.2.2 **MTT Assay of CHO-AA8 cell kill after 48 hours incubation with 0.01-50% DMSO.**

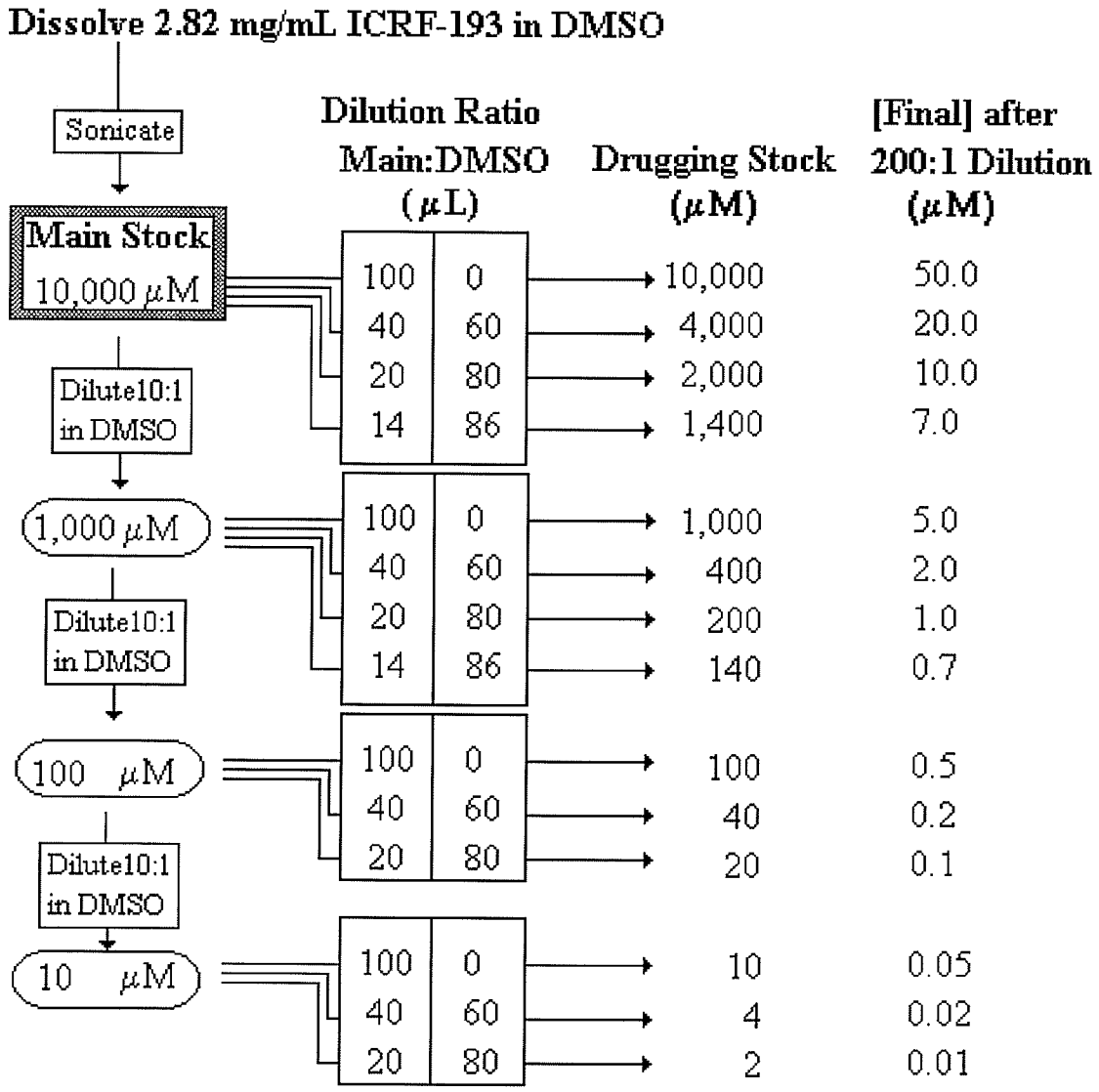


Figure 3.1.2.2.3 Drug Dilution Scheme For DMSO-Soluble Drugs

ICRF-197 was also dissolved in DMSO, but at a concentration of 20 mM, and then diluted and delivered to the wells as was the ICRF-193. The drug was delivered to the wells in 1.0 μL volumes, followed by delivery of 199 μL of αMEM. The delivery of

a 20 μM final concentration was achieved by delivering 2.0 μL of 20 mM ICRF-197 to the wells with attention to whether the resulting toxicity was due to drug or DMSO. This was verified by the curve and is described below in the results section.

3.1.2.3 Delivery of Drug to the Microtitre Plates

With exception to ICRF-193 and ICRF-197 described above, drugging procedures were the same for all of the drugs. After the 24 hours of initial incubation, drug was added to the microtitre plate wells. After the initial weighing and dissolving of the drug in medium, the drug was filtered and then diluted into cell culture medium by serial ten-fold dilution to 1/10, 1/100, 1/1000, and 1/10,000 as described. Volumes of each concentration were prepared for drugging of the cells within a certain range of drug concentrations. That is, the dilutions from 10,000 μM to 1 μM were each used to drug a certain portion of the experiment. For example, the 100 μM was used only to drug the 10-50 μM range, while 1000 μM was used for the 100-500 μM range. In the drugging, no more than 100 μL of drug or medium was added per well. All drug additions were made on the basis of a final well volume of 200 μL, including the initial 100 μL of fluid from the cell seeding. Thus, if only 50 μL of drug solution were added to a particular row of wells, the remaining volume was made up with medium to give the correct final drug concentration in a volume to 200 μL. Addition of drugs and medium was made using a 8 x 200 μL multichannel pipettor. Proper distribution of the well contents was done by mixing with the pipettor after all solutions had been added. A typical drug map is illustrated in Figure 3.1.2.3.1.

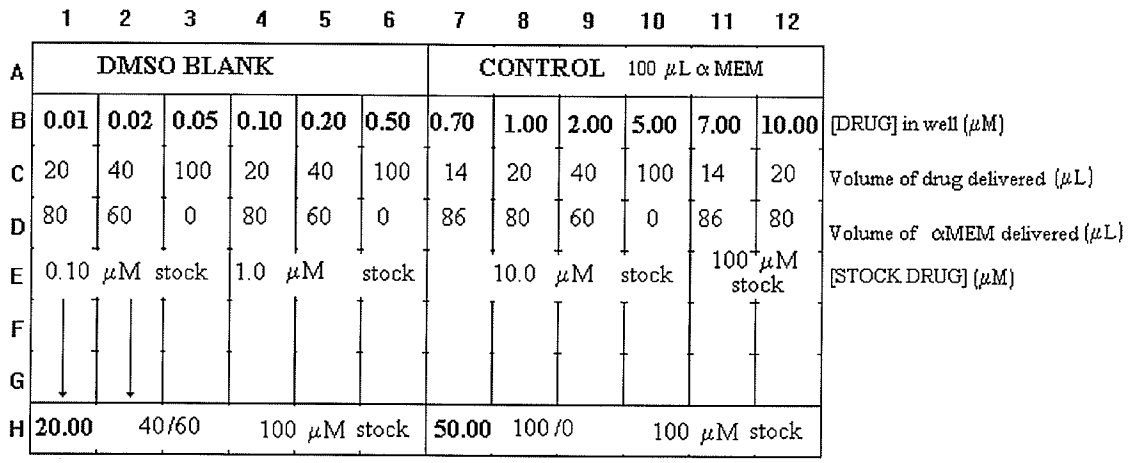


Figure 3.1.2.3.1 A Typical Drug Map For αMEM-Soluble Drugs

Use of the drug map illustrated in Figure 3.1.2.3.1 is important during the actual preparation of the experiment in order to ensure the preparation not only of the correct concentrations of drugs used, but also the correct volumes. The above scheme represents the addition of drug solutions and medium to each of the wells in a 96-well microtitre plate. The drug had to be weighed out in sufficient quantity to prepare enough drug for delivery into the wells as well as for dilutions and delivery of diluted drug into wells. Each set of experimental drug concentrations tested on a microtitre plate seeded with cells is tested in six wells and enough drug of given stock solution must be prepared for each set of wells times six. Also enough stock must be available for the 10-fold dilution. For example, beginning with 1.0 mL of 1000 μM ICRF-161, the amount of 100 μM was prepared according to the volume required for the wells as delivered per well; [(100 + 40

+ 20 + 14) μL] x 6 = 174 μL x 6 = 1044 μL. This much was required for not only delivery to those wells drugged from the 100 μM stock solution, but these volumes were also used for the 10.0, 1.0, and 0.10 μM druggings. Thus, enough of the 1000 μM solution must be diluted for use for dilution for 100 μM to 10 μM and so on. In addition to the 1044 μL required for drugging, 200 μL should be available for dilution, and so a total of 1244 μM is required. Since use of the multichannel pipettor required a slightly greater volume to fill the reservoir, 2 mL of 100 μM drug were prepared by diluting 200 μL of the 1000 μM drug solution with 1800 μL of medium. This was true for all subsequent dilutions as well.

When seeding the experiment, wells Al through A6 were left empty and were later filled with DMSO for spectrophotometer blanking in the formazan solubilizing step of the experiment. Wells A7-Al2 were seeded with cells, but these well were controls and received only 100 μL of cell culture medium and no drug. Wells B2-G2 received 20 μL of the 0.1 μM drug, B3-G3 received 40 μL of the 0.1 μM drug and so on. In each of the well sets, the drug volume added never exceeded 100 μL and if less than 100 μL was added, the remainder was made up to 100 μL using cell culture medium to give total addition of 100 μL and total well volume of 200 μL.

Addition of drug was carried out differently for ICRF-193 and ICRF-197 which were dissolved in DMSO. After the drugs were diluted into useable concentrations (Figure 3.1.2.2.3), they were delivered in 1.0 μL volumes to the existing 100 μL in the wells. Immediately after the addition of drug to one set of wells, 99.0 μL of αMEM was

delivered to each well using the multichannel pipettor. This resulted in fulfilling volume requirements for the wells as well as mixing the solution thoroughly. The drugs in DMSO were delivered to each well individually using an Eppendorff 0.5-10 μL micropipettor with sterile microtips.

3.1.2.3 Measurement of Cytotoxic Effects.

After the addition of the drug, the microtitre plates were returned to the incubator for 48 hours. After this incubation, MTT was added using the multichannel pipettor. The addition of MTT was always 10% of the final volume *i.e.* if the final volume of the wells was 200 μL including growth medium and drug, then 20 μL of 2.5 mg mL^{-1} MTT in Dulbecco's PBS was added to each well. The MTT was allowed to incubate for 4 hours in the incubator, after which all of the medium was aspirated off and the formazan crystals developed by the live cells were dissolved in 100 μL of spectrophotometric grade DMSO. Best results were obtained when the plates were allowed to sit for at least three hours prior to reading on the plate reader. The readings were made using $\lambda = A_{490}$ minus $\lambda = A_{650}$ nm.

3.1.3 RESULTS

3.1.3.1 Cytotoxicities of the Bisdioxopiperazines

Data from all cytotoxicity experiments were used to determine the 50% inhibitory concentrations for the drugs tested on CHO-AA8 cells. Using a 4-parameter nonlinear curve fitting program in Sigmaplot, 5.0 the data from the cytotoxicity assays were used

to calculate IC_{50} values for each of the assay curves where any degree of cell kill was observed. The initial determinations of the IC_{50} value are determined by substitution of the maximum absorbance, *a* and the minimum absorbance, *d* into the equation,

$$Abs_{490\ obs} = (a-d) / (1+ (x/c)^b) + d$$

where *x* is the drug concentration (μM), and *b* is the exponential factor indicating the rate at which toxicity increases with increases in drug concentration. The *c* value represents the 50% inhibitory concentration (μM) of the drug computed after the function has been fitted. Substitution of the *a* and *d* values into a 2 parameter logistic fit results in a 2 parameter fit, computing *b* and *c*. These computed *b* and *c* values from the 2 parameter fit were then resubstituted into the same equation as best guesses along with the *a* and *d* values and a more accurate 4 parameter fit was made to determine *b* and *c*. In the case of certain drugs, the higher concentrations of drug tested began to indicate toxicity, but sufficient drugs concentrations were not tested to provide a *d* value that would allow for a measurable toxicity. This occurred due to poor solubility of these drugs in cell culture medium. It was not possible to dissolve the drugs in DMSO for delivery into an aqueous environment. They would immediately precipitate out of solution in cell culture medium even though they were dissolved completely in DMSO. In these cases the *b* values were small and the *c* values inaccurate with large standard errors. To remedy these problems, the *d* value was constrained to equal 0 and a 3 parameter fit was made. This kind of calculation was done in computing values for ICRF-192, and ICRF-197 and these are indicated in table 3.1.3.1.2 by the * symbol. The curves and the values computed are

presented below for each category of agents tested. In the case of ICRF-193, the 4 and 3 parameter fits resulted in substantial errors in both the b and the IC_{50} values and so these values were computed using a 2 parameter fit where a was set to Abs_{490max} and d was set to Abs_{490min}. This is indicated on the table by the ** symbol. Where drugs failed to show any toxicity, the minimum IC_{50} values were defined as being equal to five times the maximum concentration tested (†).

Many of the drugs used exhibited poor solubility in cell culture medium and in aqueous environments in general. These were dissolved in DMSO, which mediates it own cytotoxic effects. In order to be able to utilize the potential for dissolving these poorly soluble drugs in DMSO, the window of "safe" DMSO concentration needed to be established. As mentioned in 3.1.2.2, a preliminary experiment was conducted to measure the cytotoxic effects of DMSO. The results of this experiment are illustrated in Figure 3.1.2.2.2, which shows that below 0.9% DMSO (v/v), no DMSO-mediated cytotoxicity was observed.

Table 3.1.3.1.1 lists the IC_{50} values for this series of drug toxicity experiments as well as the exponential factors computed by the fitting. Following the table are Figure 3.1.3.1.1 showing the structures of the ICRF-154, ICRF-159, ICRF-161, ICRF-192, ICRF-193, ICRF-197 and ADR-559. Figure 3.1.3.1.2 shows ICRF-186, ICRF-187 its intermediate hydrolysis products, **B** and **C** as well as the final hydrolysis product, ADR-925. Figure 3.1.3.1.3 illustrates the structures of the BLDP Series bisdioxopiperazines. Figures 3.1.3.1.4 through 3.1.3.1.18, illustrate the cytotoxicity profiles of each of the

bisdioxopiperizines. Compounds **B**, **C**, and ADR-925 showed no true IC_{50} values however, linear regressions of their cytotoxicity profiles show that drug doses could not be correlated to cytotoxicity. Slopes of each of these lines were computed using the linear regression function within the Sigmastat program. The column numbers representing the drug concentrations used in the cytotoxicity experiment were selected as the independent variable and the corresponding absorbance, indicating the degree of cell survival, was selected as the dependent variable. A linear regression calculation for compound **B** gave a slope of $-7.9 \times 10^{-5} \pm 7.9 \times 10^{-5}$ indicating that the slope of the curve was conceivably zero. The P value of 0.341 indicates that the relationship between the drug concentration and the resulting toxicity was not significant. The same calculation for compound **C** gave a slope of $-6.8 \times 10^{-5} \pm 12.2 \times 10^{-5}$ and a $P = 0.587$. The slope is again zero for all intents and purposes and the relationship between drug dose and cytotoxicity is not significant. The slope calculation for the final hydrolysis product of ICRF-187, ADR-925 had a slope of $-2.3 \times 10^{-5} \pm 19.3 \times 10^{-5}$ with a P value of 0.907. Again the slope is zero and the relationship is not significant.

Overall, the intermediate and final hydrolysis products of ICRF-187 are not regarded as toxic toward cells, even at doses as high as 200 μM. For this reason the minimum IC_{50} values for each of these compounds was defined as being equal to five times the highest dose tested.

Table 3.1.3.1.1

IC_{50} Values For Bisdioxopiperazines Incubated for 48 Hours With CHO-AA8 Cells

DRUG	IC_{50} (μM)	SEM (μM)	% CV	Exponential Factor	SEM	% CV
ICRF-154	11.6	1.7	14.7	7.0	6.0	87
ICRF-159	2.7	0.5	17	2.5	0.7	30
ICRF-161	14.4	2.5	17.4	3.7	1.7	46
ICRF-186	3.0	0.6	20.0	3.5	0.8	22
ICRF-187	2.7	0.2	7.0	2.6	0.5	17
ICRF-192*	340.2	106.2	31.3	1.4	0.5	31
ICRF-193‡	0.019	0.002	9.8	5.6	3.4	59
ICRF-197*	11.4	0.5	4.4	3.27	0.42	12.8
ADR-559**	17.2	3.7	20.7	1.6	0.2	22
ADR-925†	> 1000	0	0	ND	ND	ND
B†	> 1000	0	0	ND	ND	ND
C†	> 1000	0	0	ND	ND	ND
BLPDG2A	745	54	7.3	3.1	0.7	23
BLPDB2†	> 300	0	0	ND	ND	ND
BLPDC4	69.8	6.8	9.7	3.1	0.7	23
BLPDE1	485	208	42.5	1.9	0.7	40

* 3-parameter fit was used where *d* was constrained to 0.

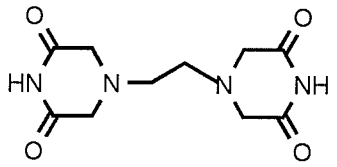
‡ 2-parameter fit where $a = Abs_{490max}$, d = Abs_{490min} and *b* and IC_{50} are computed.

** 3 parameter fit where first 7 concentration values were averaged to give an *a* value and *d* was constrained to 0.

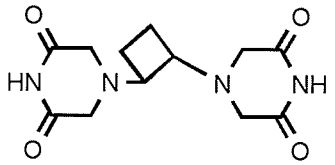
† Drug was not toxic and thus, no true IC_{50} or *b* value were determined (ND). Minimum IC_{50} was defined as being equal to five times the maximum drug concentration tested.

Figure 3.1.3.1.1

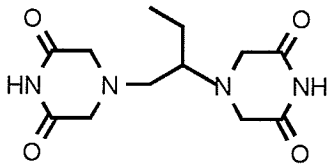
The ICRF Series Bisdioxopiperazines



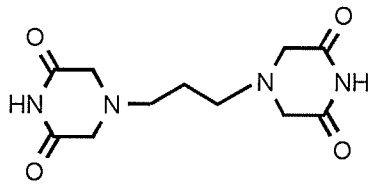
ICRF-154
Molar Mass: 254.25 g/mol
Solubility in αMEM: ~0.1 mg/mL (393 μM)



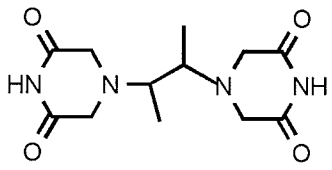
ICRF-197
Molar Mass: 280.28 g/mol
Solubility in DMSO: 5.6 mg/mL 2 0 mM)



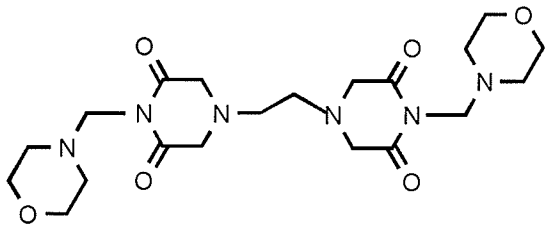
ICRF-192
Molar Mass: 282.30 g/mol
Solubility in αMEM: 0.1mg/mL (354 μM)



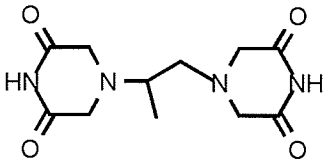
ICRF-161
Molar Mass: 268.27 g/mol
Solubility in αMEM: 1.0 mg/mL (3700 μM)



ICRF-193
Molar Mass: 282.30 g/mol
Solubility in α–MEM: <0.1 mg/mL (<350 μM)
Solubility in DMSO: >10 mM



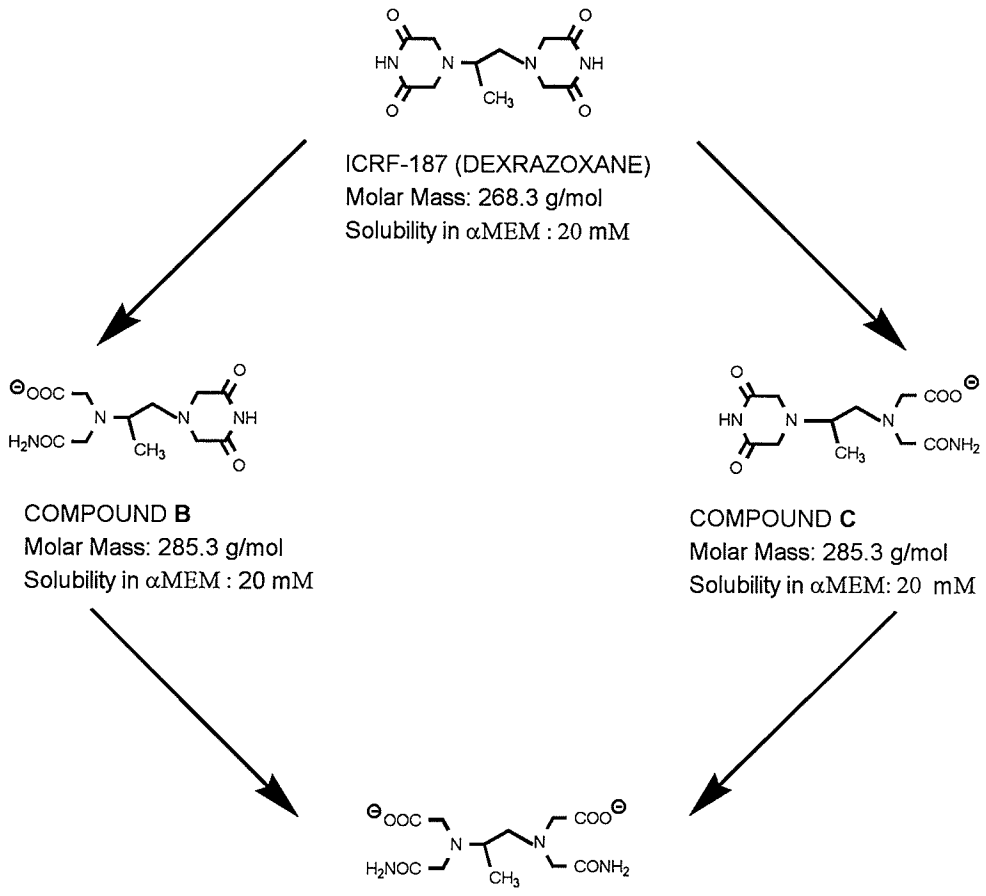
ADR-559
Molar Mass: 465.53 g/mol
Solubility in αMEM: >1.0 mg/ml (>2150 μM)

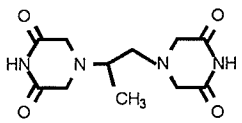


ICRF-159
Molar Mass: 268.27 g/mol
Solubility in αMEM: 20 mM

Figure 3.1.3.1.2

ICRF-186 (LEVRAZOXANE), ICRF-187 (DEXRAZOXANE, ADR-529) AND IT S METABOLIC HYDROLYSIS PRODUCTS, B, C, AND ADR-925

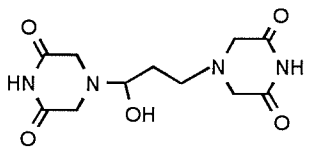




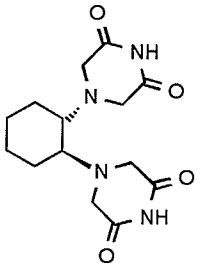
ICRF-186 (LEVRAZOXANE)
ICRF-186 IS THE (-)-(*R*) ISOMER OF ICRF-187
Molar Mass: 268.3 g/mol
Solubility in αMEM : 20 mM

Figure 3.1.3.1.3

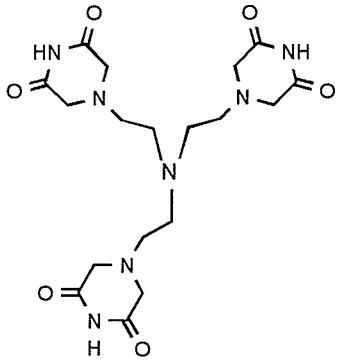
The BLPD Series Bisdioxopiperazines



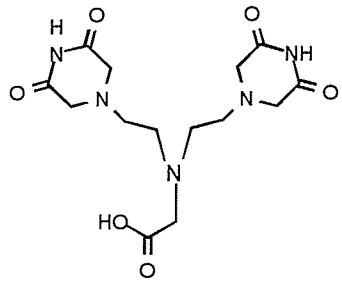
BLPD2GA
Molar Mass: 283 g/mol
Solubility in αMEM: > 1mg/mL (>3500 μM)



BLPDB2
Molar Mass: 308 g/mol
Solubility in αMEM : 0.1mg /5.15mL (63 μM)



BLPDC4
Molar Mass: 413 g/mol
Solubility in αMEM: > 1mg/mL (>2400 μM)



BLPDE1
Molar Mass: 355 g/mol
Solubility in αMEM: > 1mg/mL (>2800 μM)

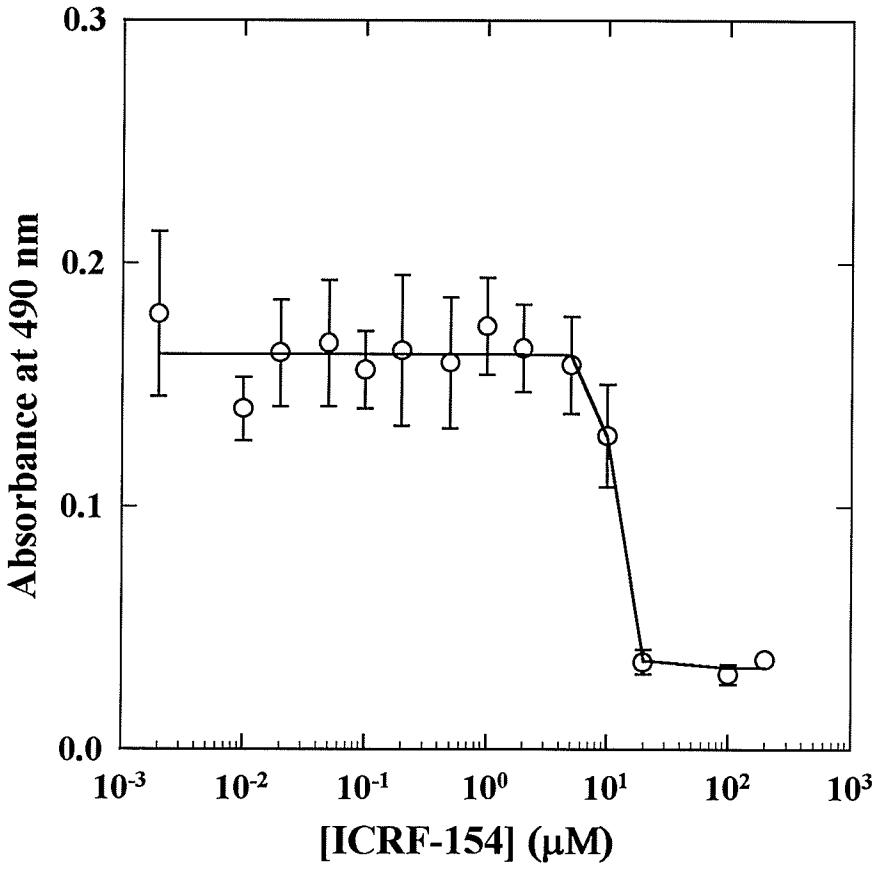


Figure 3.1.3.1.4 **MTT Assay of ICRF-154-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

NOTE: The left-most points on all cytotoxicity graphs in chapters 3, 4, and 5 of this thesis represent zero values.

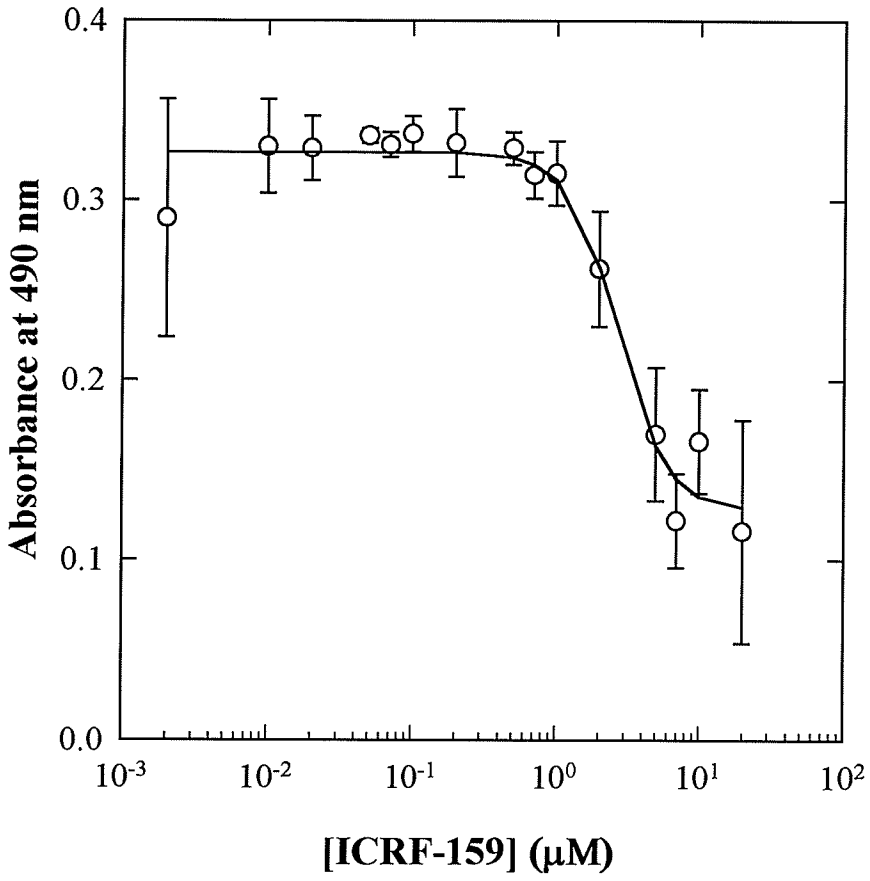


Figure 3.1.3.1.5 **MTT Assay of ICRF-159-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

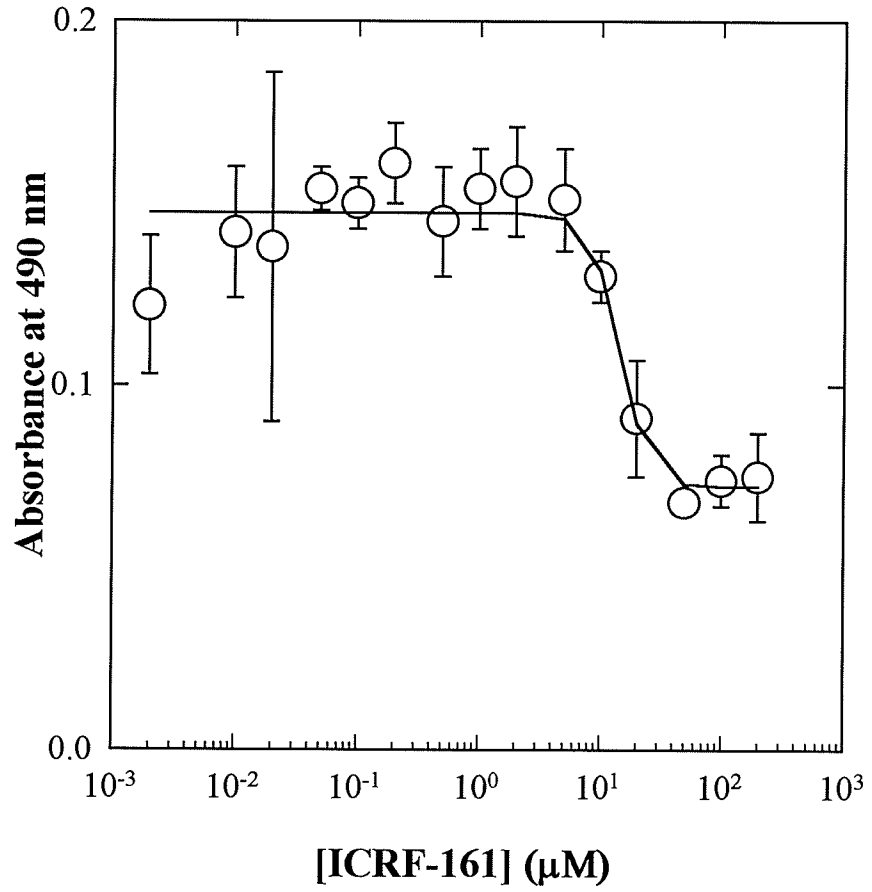


Figure 3.1.3.1.6 **MTT Assay of ICRF-161-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

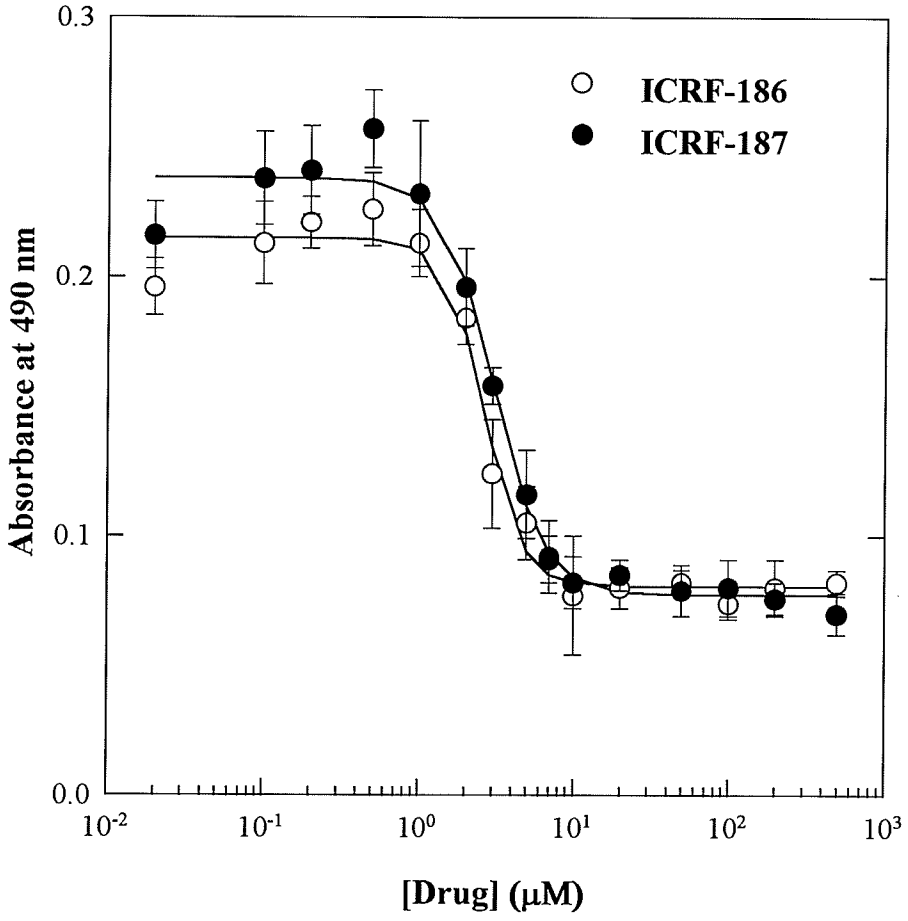


Figure 3.1.3.1.7 **MTT Assay of CHO-AA8 cell kill after 48 hours incubation with ICRF-186 and ICRF-187.**

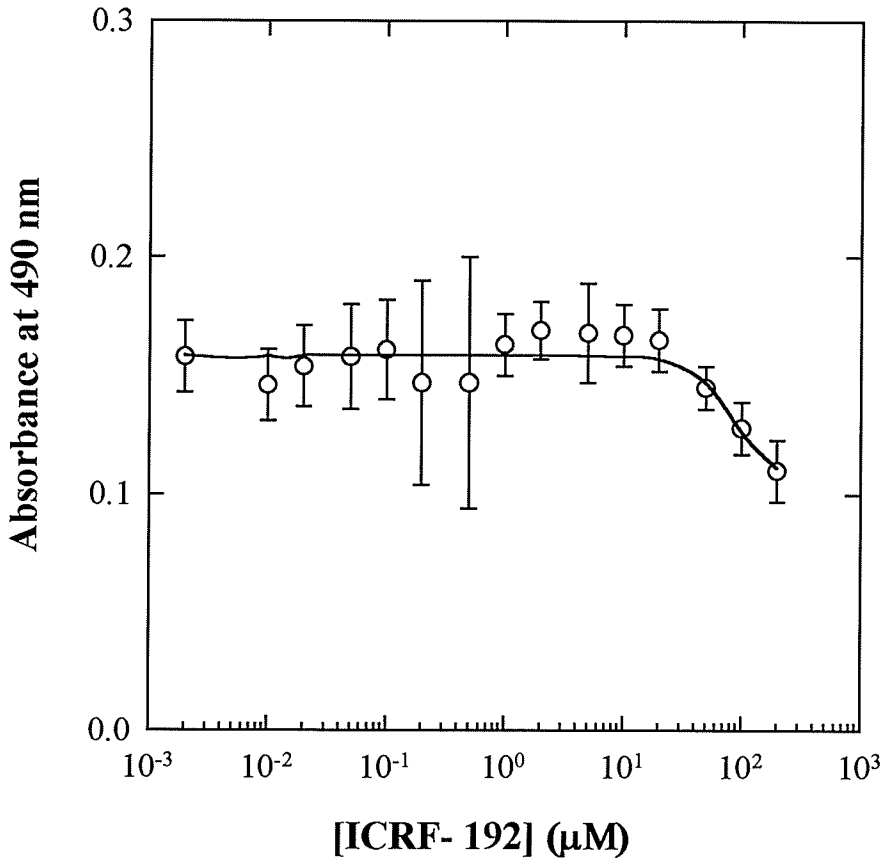


Figure 3.1.3.1.8 **MTT Assay of ICRF-192-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

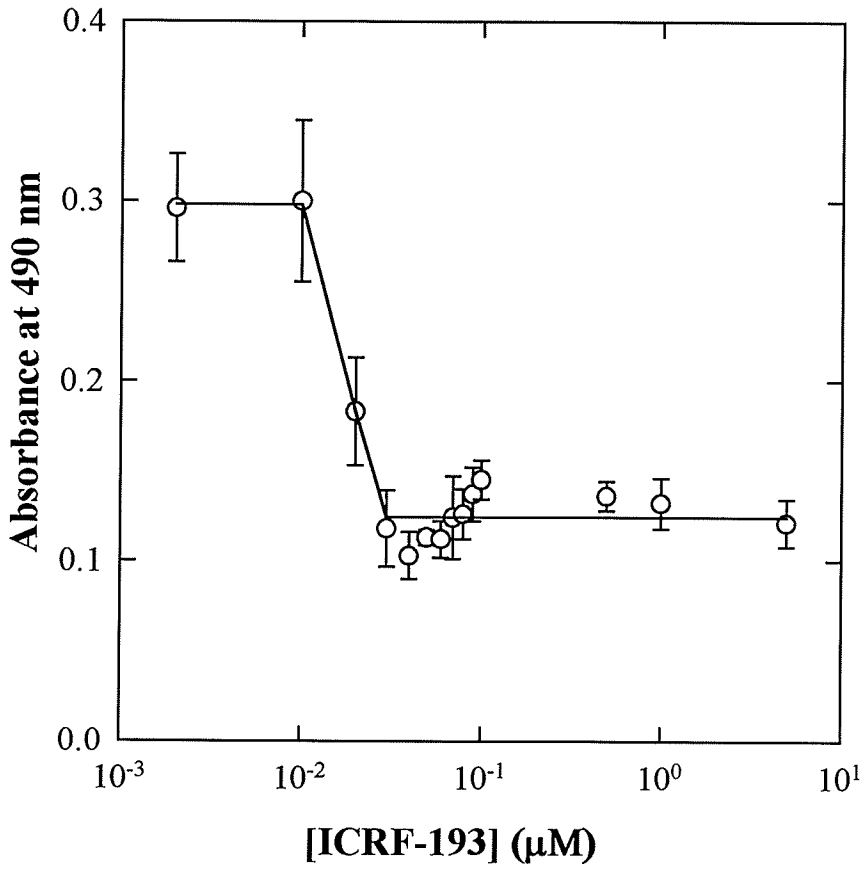


Figure 3.1.3.1.9 **MTT Assay of ICRF-193-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

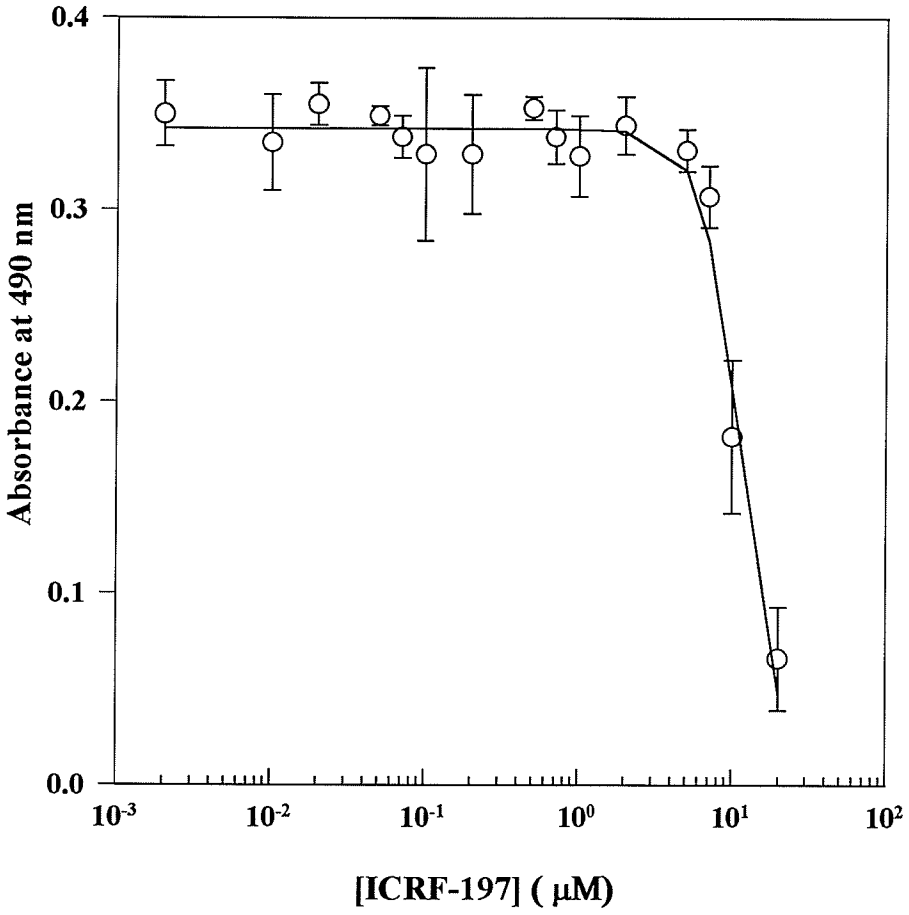


Figure 3.1.3.1.10 **MTT Assay of ICRF-197-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

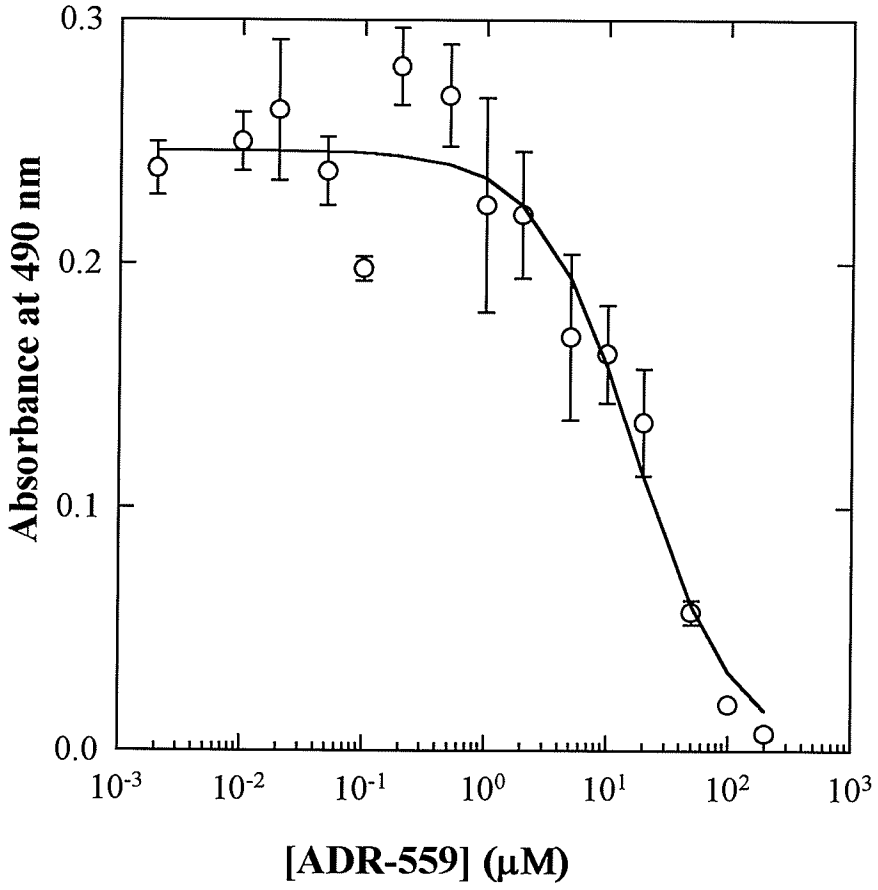


Figure 3.1.3.1.11 **MTT Assay of ADR-559-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

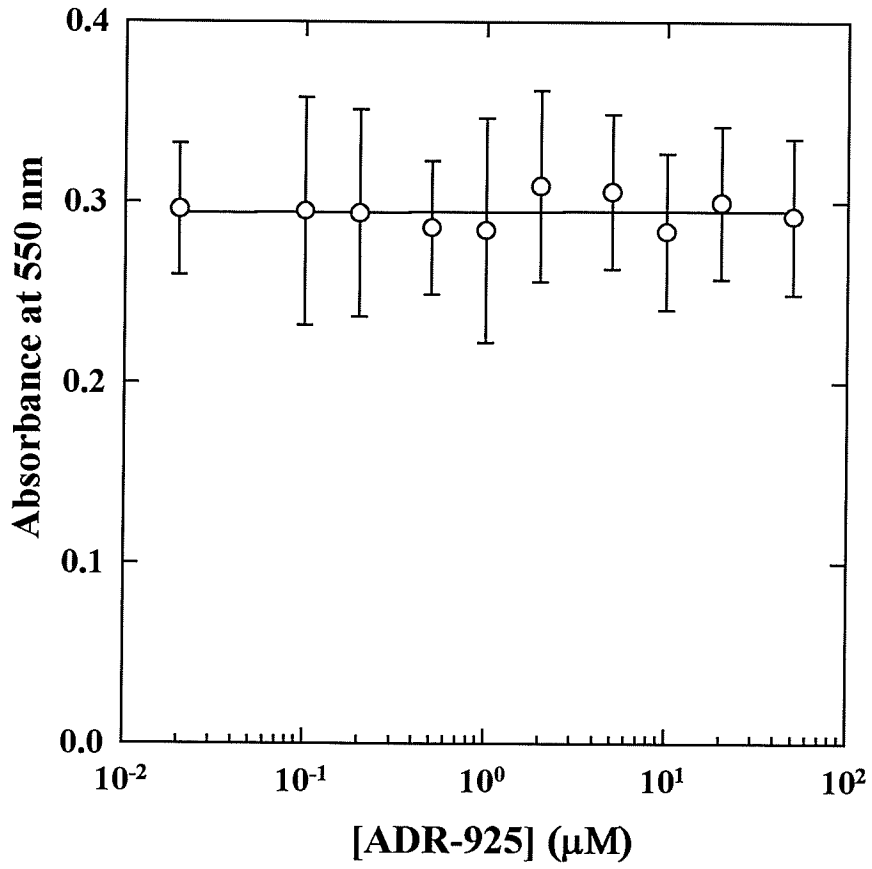


Figure 3.1.3.1.12 **MTT Assay of ADR-925-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

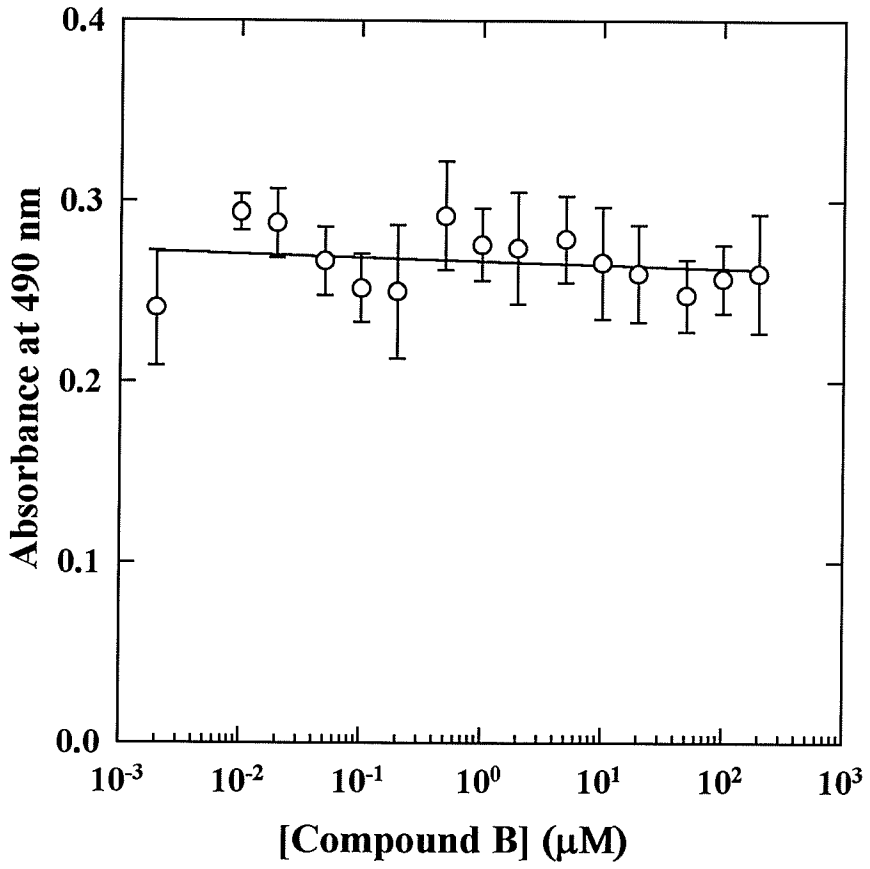


Figure 3.1.3.1.13 **MTT Assay of compound B-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

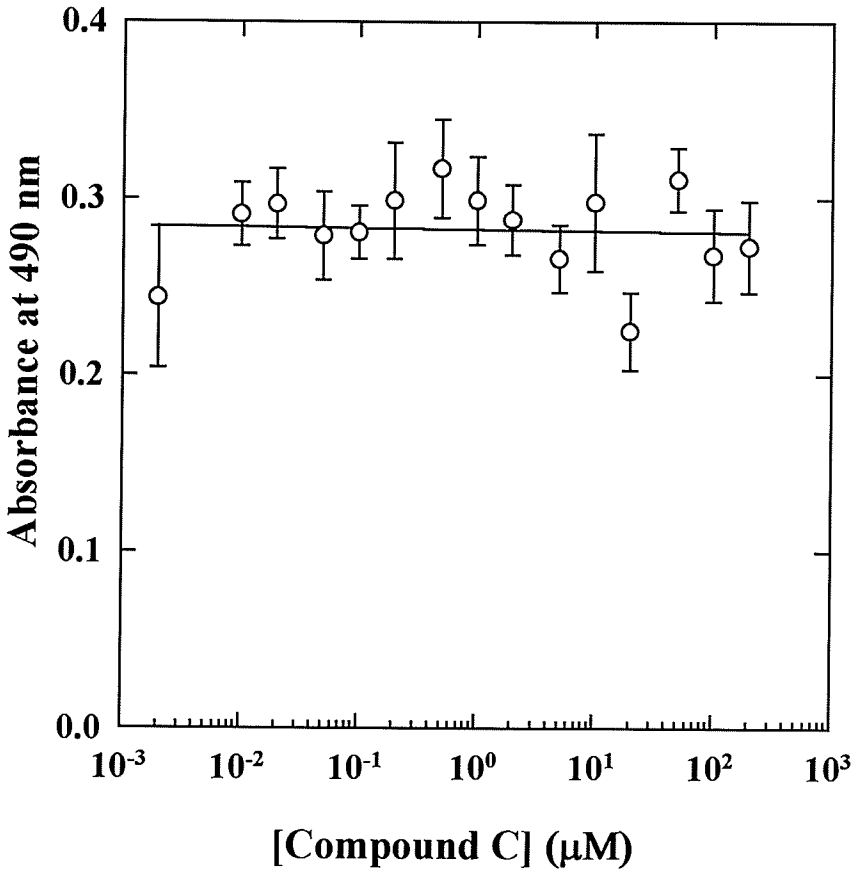


Figure 3.1.3.1.14 **MTT Assay of compound C-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

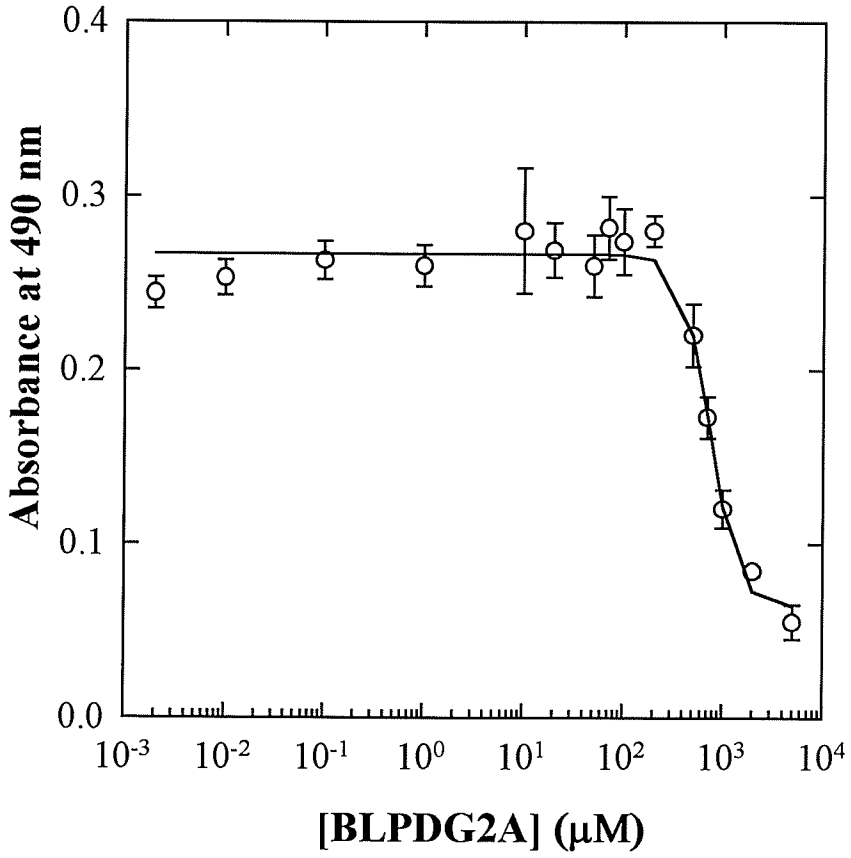


Figure 3.1.3.1.15 **MTT Assay of BLPDG2A-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

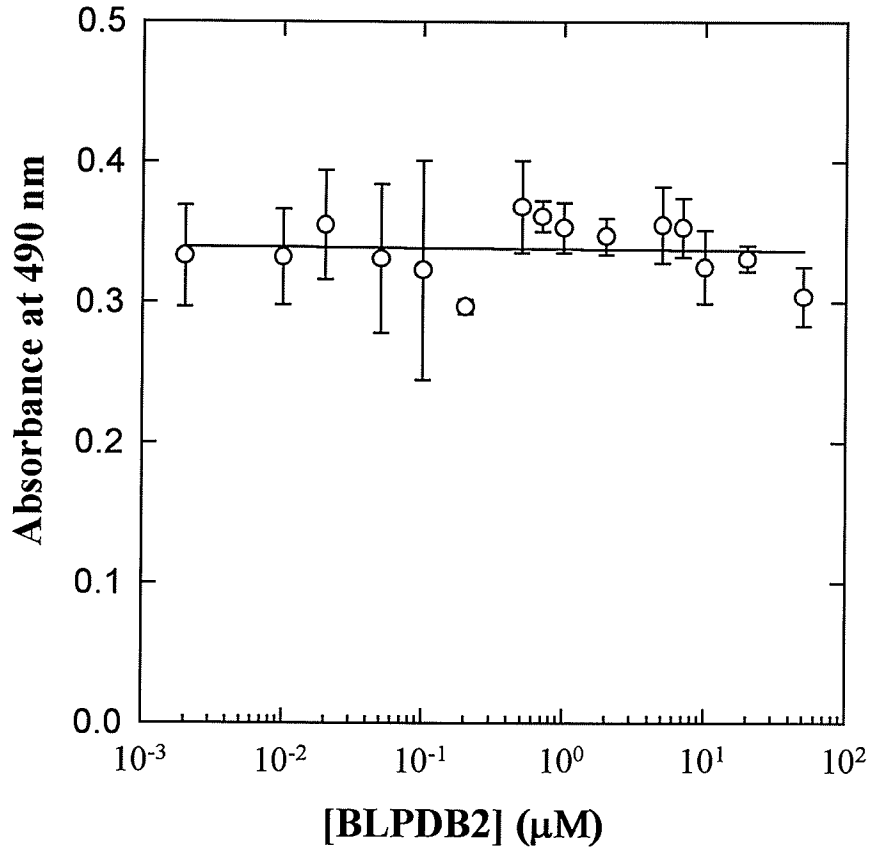


Figure 3.1.3.1.16 **MTT Assay of BLPDB2-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

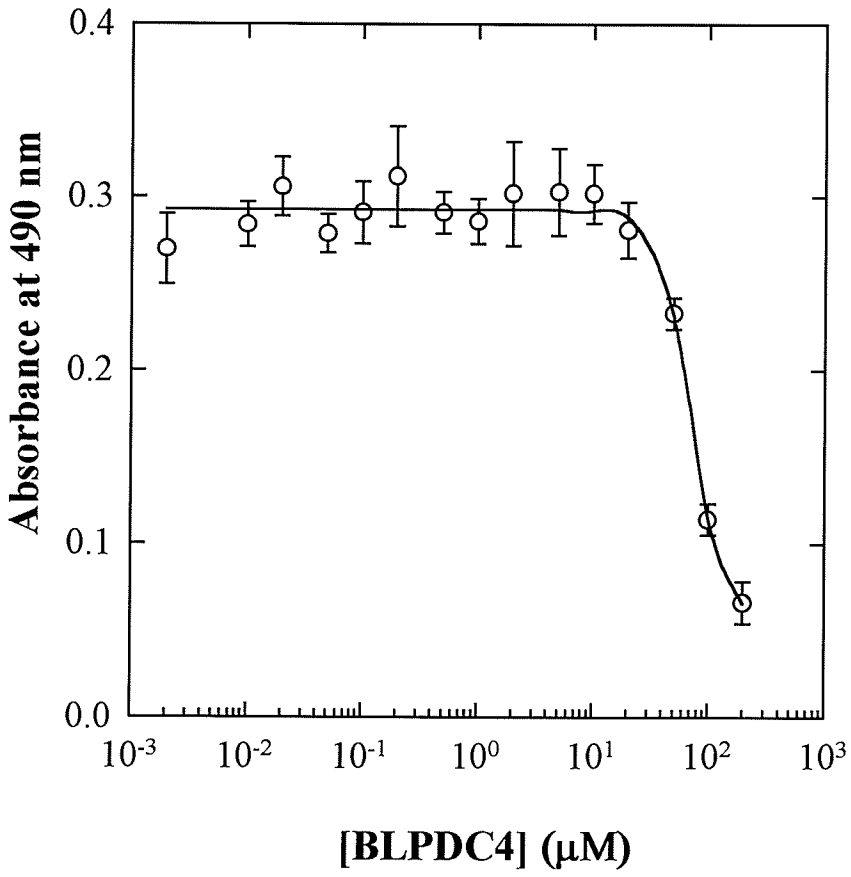


Figure 3.1.3.1.17 **MTT Assay of BLPDC4-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

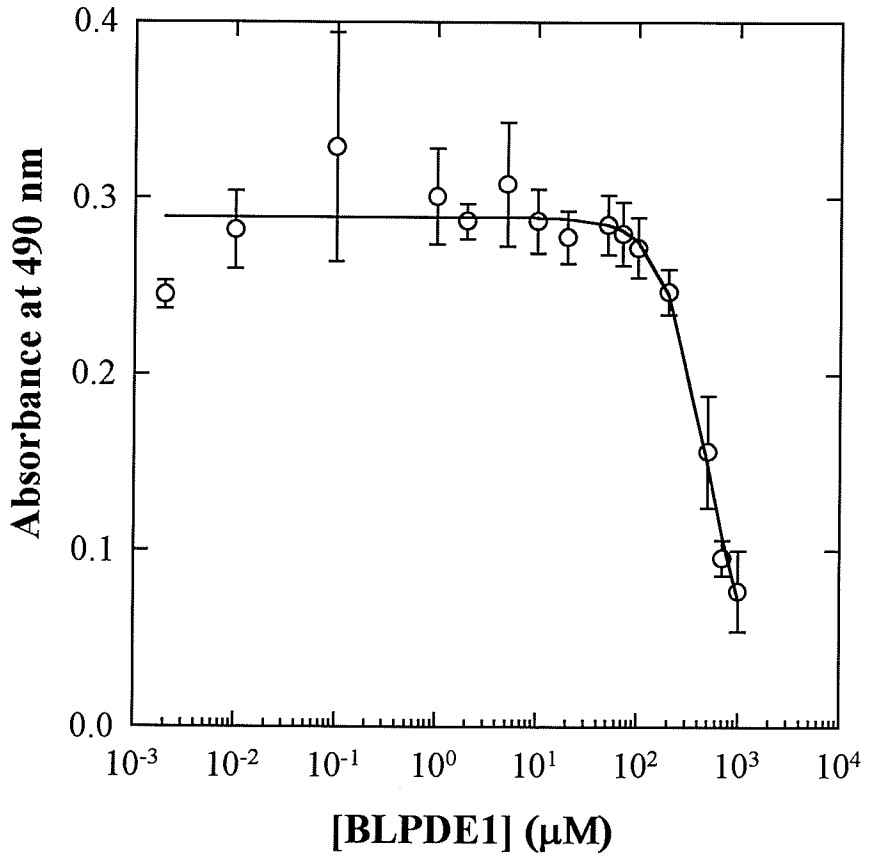


Figure 3.1.3.1.18 **MTT Assay of BLPDE1-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells.**

3.1.3.2 The Relationship Between The Octanol : Water Partition Coefficients And Cytotoxicities Of The ICRF-Series Bisdioxopiperazines

The partition coefficient of a drug is defined as the equilibrium constant

$$P = [drug]_{lipid} / [drug]_{water}$$

of drug concentrations in the two phases. These are usually measured *in vitro* using *n*-octanol as the lipid phase and phosphate buffer of pH 7.4 as the aqueous phase. The value *P* is a dimensionless ratio which expresses the polarity and lipophilicity or hydrophobic character of a molecule. This method is widely employed in structure activity studies. Drug transport characteristics, be it from the site of application to a receptor or through a cell membrane are influenced directly by partition coefficients since drugs must travel through aqueous medium, such as blood, to reach a target tissue and then must traverse cells to reach their site of action.

As is shown in examination of the numerous bisdioxopiperazine structures and comparing them to their respective degrees of cytotoxicity, there would appear to be a relationship between the structure of a given drug and its ability to kill cells. It is not clear however, whether the structural differences seen among these drugs affects the degree of toxicity simply by affecting the ability of the drug to enter the cell and cause a non-specific reaction, or whether the effect is based on interaction with an entirely different and more specific target within the cell, or both. The relationship between cytotoxicity of bisdioxopiperazines and their lipophilicities was not known and for this

reason they were compared.

Octanol:water partition coefficients have been given by Creighton *et al.*, (1979) for most of the compounds tested in this series of experiments. When the logarithm of these values was plotted against the logarithm of the IC_{50} value determined for each of these drugs, a weak relationship was found. Listed in Table 3.1.3.2.1 are the octanol:water partition coefficients and the IC_{50} values and their respective logarithms for several drugs.

The plot representing the table of log inhibitory concentrations and log partition coefficients (Log *P*) is shown in Figure 3.1.3.2.1. Linear regression gave a 0.844 coefficient of correlation and a significance, $P = 0.017$ indicating that cytotoxicity is significantly related to the octanol:water partition coefficient. Hence, the ability of the drugs to permeate the cell membrane to varying degrees is related to their toxicities.

Table 3.1.3.2.1

Comparison Between Octanol:Water Coefficients of Several ICRF Compounds And Their 50% Inhibitory Concentrations After 48 Hour Incubation With CHO-AA8 Cells

DRUG	IC_{50} (μM)	Log IC_{50}	Log *P*
ICRF-154	11.6	1.06	-2.34
ICRF-159	2.7	0.431	-1.85
ICRF-186	2.7	0.431	-1.85
ICRF-187	3.0	0.477	-1.85
ICRF-192	80.9	1.91	-2.12
ICRF-193	0.019	-1.72	-0.78
ICRF-197	11.4	1.06	-1.51

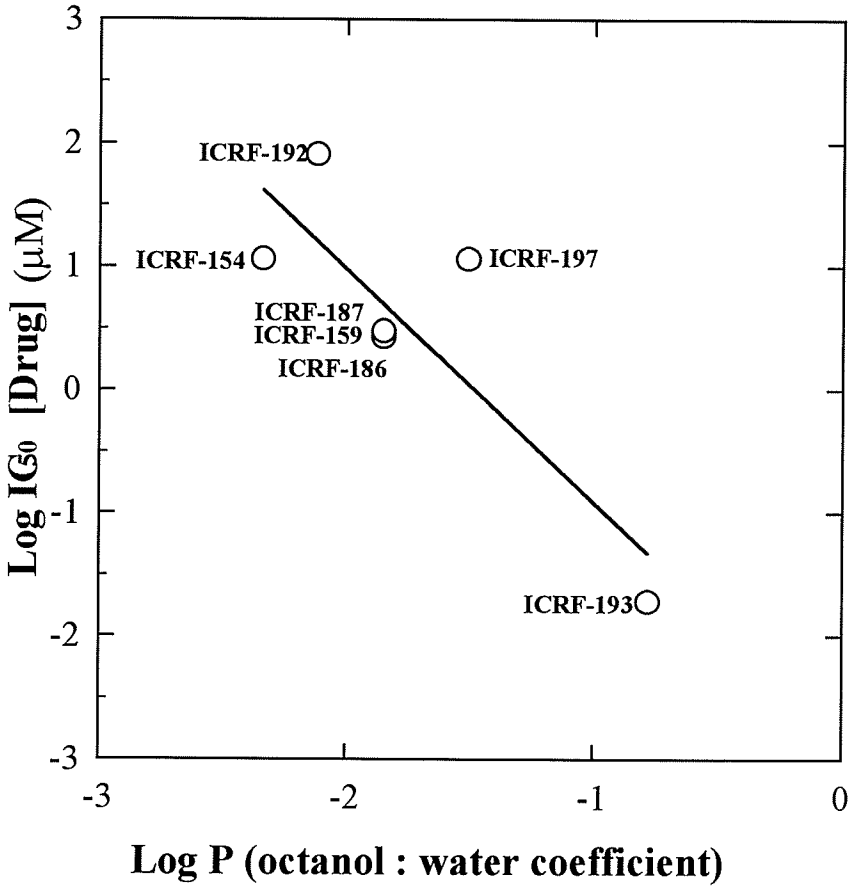


Figure 3.1.3.2.1

The Relationship Between Lipophilicity And Toxicity To CHO-AA8 Cells.

This graph shows the correlation between lipophilicity as determined by octanol:water coefficient measurements and cytotoxicity determined by 48 hour incubation of CHO-AA8 cells with several of the bisdioxopiperazines.

3.1.3.3 Bisdioxopiperazine Toxicity Related to Topoisomerase II Inhibition

Bisdioxopiperazines are known to cause cytotoxic effects which are the result of interruption of specific enzyme functions at the subcellular level. ICRF-159, ICRF-187 (Gorbsky, 1994), MST-16 (Ohno, *et al.*, 1992), ICRF-154, ICRF-193, (Tanabe *et al.*, 1991), have shown that to varying degrees, all of the bisdioxopiperazines tested showed interruption of topoisomerase II activity and subsequent growth inhibition or death. Decatenation experiments were conducted with ICRF-154, ICRF-159, ICRF-186, ICRF-187, ICRF-192, ICRF-193, ICRF-193, ADR-925, compounds **B**, and **C**, as well as the BLPD series drugs by Yalowich *et al.*, (unpublished) in order to measure the inhibitory effects of these drugs on topoisomerase II. The log IC_{50} values computed from toxicity experiments conducted with CHO-AA8 cells were plotted against log IC_{50} topoisomerase II inhibition values measured by Yalowich *et al.* These data are listed in Table 3.1.3.3.1 and a coefficient of correlation plot is shown in Figure 3.1.3.3.1.

The comparison shows the relationship between the IC_{50} values of the drugs tested on CHO-AA8 and the IC_{50} value for topoisomerase II inhibition. The coefficient of correlation calculated on the linear regression function of the Sigmastat program was 0.887 and $P < 0.001$, indicating that the relationship between the two sets of data is significant.

Table 3.1.3.3.1

Relationship Between Bisdioxopiperazine IC_{50} After 48 Hour Incubation With CHO-AA8 Cells And Percent Inhibition Of Topoisomerase II Activity

DRUG	IC_{50} (μM)	Log IC_{50} (μM)	IC_{50} Topo II Inhibition (μM)	Log IC_{50} Topo II Inhibition (μM)
ICRF-154	11.6	1.06	12.2	1.09
ICRF-159	2.7	0.431	13.4	1.13
ICRF-161	14.4	1.16	32.4	1.51
ICRF-186	2.7	0.431	12.3	1.21
ICRF-187	3.0	0.477	16.2	1.09
ICRF-192	80.9	1.91	30.4	1.48
ICRF-193	0.019	-1.72	1.0	0
ICRF-197	11.4	1.06	28.8	1.46
BLPDG2A	742	2.87	38.6	1.57
BLPDB2	> 320	2.51	20.0	1.30
BLPDC4	69.8	1.84	51.9	1.72
BLPDE1	485	2.69	65.3	1.81

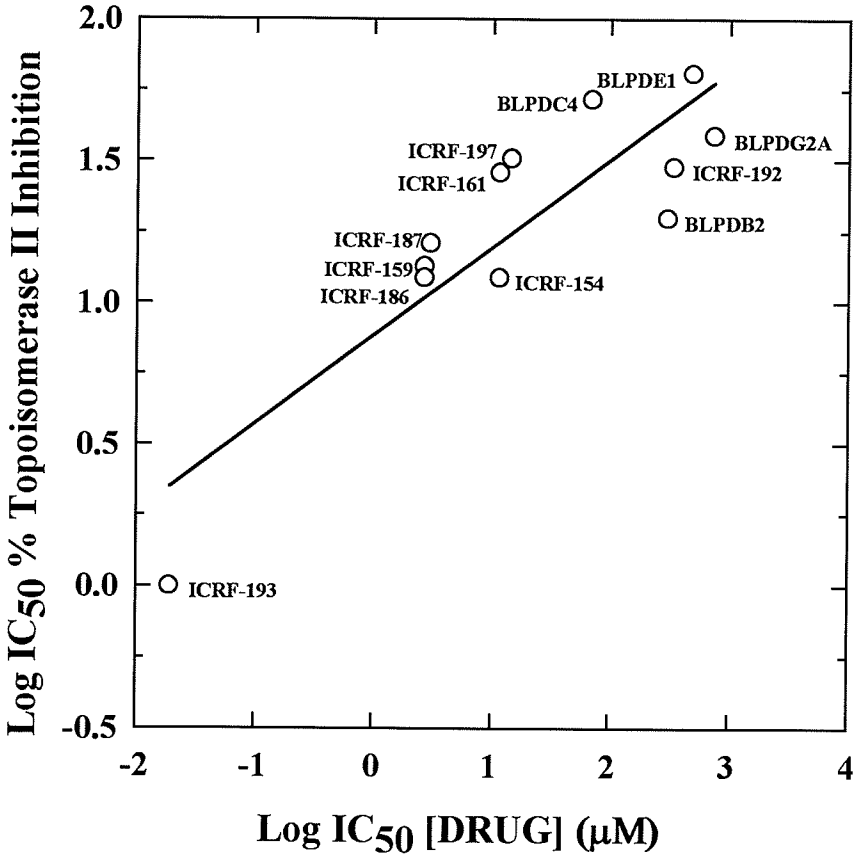


Figure 3.1.3.3.1 **Relationship Between IC_{50} and Inhibition of Topoisomerase II Activity After 48 Hour Drug Incubation With CHO Cells.**

3.1.4 DISCUSSION

The bisdioxopiperazines were developed by ICRF Laboratories in the late 1960's and early 1970's as a class of chemotherapy agents. The ICRF-series drugs first showed promise as antitumor agents in which metal chelation played a role in the tumorostatic process. The potent chelating agent, EDTA was shown to be ineffective against tumors, and this was a source of a great deal of doubt where chelation properties and antitumor activity was concerned. However, unlike the polar EDTA molecule, the ICRF series drugs were not restricted in their ability to cross membranes. The insight into the potential for metal chelation in antitumor agents resulted in the synthesis of a number of EDTA analogues and among the first effective cytotoxic agent with chelating potential was ICRF-154. ICRF-154 and the ICRF series drugs that followed were able to traverse the membrane as relatively non-polar and uncharged molecules and then hydrolyze to a metal-chelating form, which was shown to be cytotoxic (Creighton *et al.*, 1979).

The first descriptions of the cytostatic properties of one drug, ICRF-159 was by Carter (unpublished in Creighton, *et al.*, 1979). Carter observed that although mouse L cells required a dose of at least 100 $\mu g\ mL^{-1}$ (373 μM) to arrest cell division, lower doses interrupted the process sufficiently so that he saw that as the daughter cell tried to move apart during mitosis, they were held back by strands of nuclear material. The result was that the daughter cells rejoined to form a tetramer, or they separated unevenly. In either case the progeny were not viable. Experiments with BHK-21S cells showed that they have a tendency to not complete cytokineses and cells eventually become multinucleate.

Hellmann and Field (1970) showed that doses as low as 10 μg mL^{-1} (37 μM) at a brief period of time prevented PHA-stimulated lymphocytes from progressing into metaphase. Creighton's work showed that the drugs had two principle effects on cultured cells. (PHA = Phytohemaglutanin, which is a lectin from the Red Kidney Bean, *Phaseolus vulgaris*, which causes blastogenesis.) The first effect was a delay in progression of the cell cycle, though this was reversible. The second was the effect on the viability of the cell. This is discussed further in section 3.2, which deals with timed drug replenishment experiments.

In terms of structure activity relationships of the ICRF series bisdioxopiperazines, little change in the structure from the basic ICRF-154 molecule can be tolerated without loss of cytotoxic activity. First, the dioxopiperazine ring must be intact and second, the central chain may be substituted with ligands of correct configuration, but not lengthened (Creighton, *et al.*, 1979).

The experiments conducted with the ICRF series bisdioxopiperazines as well as with the BLPD series agents shows that the degree of toxicity of the agents tested varies with structure, as is expected. The range of toxicity for the drugs tested was as low as 0.019 μM and as high as 742 μM. All of these drugs contained dioxopiperazines (or their metabolic products, as in the case of ICRF-187) and small differences in their structures affected their cytotoxic capacities quite significantly. The inferences of these structural changes with respect to the resulting cytotoxicities is not understood. Though the IC_{50} value obtained by Creighton *et al.*, (1979) were often different, the trends in toxicity were not significantly different from those obtained in cell culture experiments with CHO cells.

This is likely due to the differences in experimental conditions, since Creighton's team performed *in vivo* experiments with mice. Those compounds regarded as highly cytotoxic in mice (such as ICRF-193), were found to be highly toxic in the experiments conducted with CHO-AA8 cells as well. The same pattern is true for the compounds found to be ineffective in killing cells.

In order to clarify these relationships, comparisons were made between experimental data acquired through the above cytotoxicity experiments and data regarding membrane permeability and enzyme inhibition effects. The logarithm of the IC_{50} values measured for the drugs were plotted against Log P (octanol:water coefficients), with the expectation that there would be a significant relationship between cytotoxicity and lipophilicity; however there is only slightly more that one chance in ten that there is a significant relationship. The cytotoxic properties of the bisdioxopiperazines were compared with the ability of these same drugs to inhibit the function of topoisomerase II in a cell free system. These comparisons gave significant insight into the possible mechanism of toxicity mediated by the bisdioxopiperazines. The compounds, their possible mechanisms of toxicity, were determined, and their degrees of toxicity are discussed below in greater detail.

3.1.4.1 Cytotoxicity Of The ICRF and BLPD Bisdioxopiperazine Compounds: Structure Activity Comparisons

The cytotoxicity of the bisdioxopiperazines tested in 48 hour incubations with CHO-AA8 cells is discussed in this section and make reference to structural features of the individual molecules. The structures of these compounds are given as follows: Figure 3.1.3.1.1 shows the ICRF series bisdioxopiperazines which includes the methylmorpholino derivative, ADR-559. The structure and metabolic hydrolysis of ICRF-187 (and ICRF-186) to intermediate compounds **B**, **C**, and final product, ADR-925 are shown in Figure 3.1.3.1.2. The BLPD compounds are illustrated in Figure 3.1.3.1.3.

The most toxic of the general ICRF-series group was shown to be ICRF-193 (0.019 ± 0.003 μM), which appeared to be approximately 600 times as toxic as the next most toxic compound, ICRF-154 (11.6 ± 1.2 μM). The former carries two methyl groups on its propane backbone, while the latter is saturated with hydrogen. A compound structurally similar to ICRF-154 is ICRF-161, which is not substituted in any way, but has a butane rather than a propane backbone. No information was provided by Creighton regarding the actual IC_{50} of ICRF-161, which carries a lengthened backbone, but the IC_{50} value determined in experiments with CHO-AA8 cells shows an IC_{50} value of 14.4 ± 2.5 μM, which is only slightly higher than that of ICRF-154. Experiments with ICRF-186 and ICRF-187 gave IC_{50} values very comparable to those provided by Creighton. The values obtained were 3.0 ± 0.6 μM for ICRF-186 and 2.7 ± 0.2 μM for ICRF-187, comparing well with the values given by Creighton *et al.*, (1979) of 3.0 μM for both compounds

used in *in vivo* experiments with mice. This suggests that the two isomers of the drug are not distinguished and that membrane passage is not stereospecific. Far less toxicity was manifested by ICRF-192 (340.2 ± 106.2 μM), which carries an ethyl group on its propane backbone. ICRF-197 (11.4 ± 0.5 μM) which carries an cyclobutyl group on its propane backbone, was not as cytotoxic as ICRF-187, but was comparable in toxicity with ICRF-154 and ICRF-161. The IC_{50} value of ADR-559 was computed as 17.2 ± 3.7 μM. Its structure is like ICRF-154 except that in carries a methylmorpholino group on each of the dioxopiperazine rings. ICRF-159, the racemate of ICRF-187 was less soluble than either of the isomers, and in terms of drug effect, it had an IC_{50} value of 0.407 ± 0.025 μM.

Compounds **B**, **C**, and ADR-925 (the hydrolysis products of ICRF-187) do not appear to have any significant cytotoxic effects. Cytotoxicity experiments using concentrations as high as 200 μM showed linear plots with slopes close to zero for compounds **B**, **C**, and ADR-925. The minimum IC_{50} values were defined as being equal to five times the highest drug concentration tested, *i.e.* greater than 1000 μM. The 4 parameter fit computed for BLPDG2A gave an IC_{50} value of 745 ± 54 μM. The minimum IC_{50} value of BLPDB2 was defined as being greater than 300 μM. BLPDC4 showed an IC_{50} value 69.8 ± 6.8 μM and BLPDE1 had an IC_{50} value of 485 ± 208 μM. Though, there is no data such as octanol:water partition coefficients to describe any relationship between the structures of the drugs and their membrane permeability which might influence toxicities, review of the structures illustrated in Figure 3.1.3.1.3 suggests that the compounds which possess additional polar groups, such as the hydroxyl group in the

case of BLPDG2A, and the ethanoic acid group in the case of BLPDE1, are less toxic than their less polar counterparts. Why these structural features make them less toxic is not known.

3.1.4.2 The Octanol:Water Partition Coefficients And Cytotoxicities Of The ICRF-Series Bisdioxopiperazines Incubated For 48 Hours With CHO-AA8 Cells

Water comprises a large proportion of all living systems and for this reason, all biological reactions are based on small molecules which are dissolved in the liquid phase, or alternatively on macromolecules dispersed in this phase. Most often it is a combination of both phenomena. Non-aqueous structures within a cell, such as the plasma membrane or the membranes of organelles are comprised of lipids and and are able to dissolve polar or non-polar hydrophobic molecules. It is because of the solubility characteristics of the aqueous phase and lipid-based materials that the most important physical property of pharmacologically important molecules is their solubility. In order for a drug to interact with cellular and subcellular components which carry drug receptors and thereby trigger a response, these drugs must be in solution. In theory there are no compounds which are completely insoluble. Rather, every molecule is soluble in both the aqueous and non-aqueous lipid "compartments" of a cell and the degree of solubility of the drug differs in each of these compartments. The ratio of the solubilities of a drug in these compartments is known as the partition coefficient and is a function of numerous molecular parameters (Nogrady, 1988).

The range of toxicity exhibited by the ICRF and BLPD groups of bisdioxopiperazines suggests that there is structural relationship to the ability of the drug to inflict damaging effects upon the cell. Being fairly small molecules, minor structural changes result in profound changes in the cytotoxic effects mediated by the drugs. Speculation over whether or not their structural changes had direct effects on the drugs' respective abilities to permeate cell membranes and thereby influence the potential for cytotoxicity lead to examination of octanol:water partition coefficient experiments conducted by Creighton *et al.*, in 1979. Plotting of logarithm *P* (octanol:water partition coefficients) values against the log of the IC_{50} values computed for the ICRF series drugs tested, resulted in the illustration showed in 3.1.3.2.1. The linear regression calculation indicated a coefficient of correlation, *R* equal to 0.844. The significance value, $P = 0.017$ indicated that there is a degree of significance in this correlation. From the apparent differences in the IC_{50} values recorded it would seem plausible that membrane permeability based on structure is significantly related to the toxicities, and based on the coefficient of correlation it is reasonable to conclude that membrane permeability plays a significant role in the cytotoxic effects of the drugs tested in this series of experiments.

3.1.4.3 Bisdioxopiperazine Toxicity Related to Topoisomerase II Inhibition

One of the enzymes involved in the DNA replication process is topoisomerase II. In general, topoisomerases are nuclear enzymes which function to resolve topological problems which are associated with DNA transcription and translation. These problems

are usually overwinding, underwinding and catenation of the DNA strands. Topoisomerase II, especially that found in eukaryotic cells is responsible for relaxing both negatively and positively wound DNA supercoils, catenation and decatenation, as well as knotting and unknotting of DNA (D'Arpa and Liu, 1989). There are two topoisomerase II forms known in mammalian cells. Topoisomerase IIβ is expressed at low and constant levels throughout the cells cycle and does not change in periods of cell proliferation. Topoisomerase IIα is expressed at high levels during periods of cell proliferation. This form is regulated by the cell cycle. (Gorbsky, 1994).

Experiments were conducted by (Yalowich *et al.*, unpublished) which defined the potential of a number of the bisdioxopiperazines to inhibit topoisomerase II activity in a cell free system. The drugs tested were ICRF-154, ICRF-159, ICRF-161, ICRF-186, ICRF-187, ICRF-192, ICRF-193, ICRF-197, BLPDG2A, BLPDC4, BLPDE1, and BLPDB2. Experiments with ICRF-193, the most potent of the bisdioxopiperazines, on topoisomerase I show that the effects manifested by these agents do not affect this enzyme (Tanabe, 1991). ICRF-159 and ICRF-187 are reported to affect the G_2/M phase boundary and are able to inhibit topoisomerase II without the formation of cleavable DNA-enzyme complexes (Gorbsky, 1994). Clarke *et al.*, (1993) report this to be the case for ICRF-193 as well.

The results of the experiments performed in this chapter were correlated with decatenation and topoisomerase inhibition data provided by Yalowich *et al.*, (unpublished). The comparison of these data shows a strong coefficient of correlation (*R*

= 0.887). The significance of $P < 0.001$ indicates that toxicities manifested by the ICRF and BLPD series bisdioxopiperazines are significantly related to the topoisomerase II inhibition shown for the drugs. This suggests that the mechanism of action of the bisdioxopiperazines is likely to be significantly related to the inhibition of topoisomerase II activity. This agrees with the findings of the above-mentioned investigators however, the actual mechanism of the drug with respect to topoisomerase II is not known.

Examination of the cytotoxicity generated in the CHO-AA8 cell experiments and comparison with data given by Creighton *et al.*, (1979) show the cytotoxicities of both ICRF-186 and ICRF-187 to be the same, within the standard error of the mean. This indicates that there was no difference between the effects of the drug *in vivo* with mice or *in vitro* with CHO cells. Similarly, lipophilicity experiments showed the membrane permeability of the two isomers to be equal. The effects mediated by ICRF-186 and ICRF-187 on topoisomerase II were also found to be the same, suggesting that there is no stereospecific preference where inhibition of the topoisomerase enzyme is concerned.

3.2 ICRF-187 REPLENISHMENT EXPERIMENTS

3.2.1 INTRODUCTION

In addition to the singular drug effects of ICRF-187, replenishment drugging experiments were conducted to determine whether or not replacement of the rapidly hydrolyzed parent drug had any cytotoxic effects in addition to those observed in a single drugging over the same time frame. The most rapid degradation occurs in the metabolic

hydrolysis of ICRF-187 to Compound **B** and the half time of hydrolysis is minimally 9.3 hours in a solution at 37° and pH 7.4 (Hasinoff, B.B., 1993) The metabolic products **B** and **C** are more polar than ICRF-187 (Figure 3.1.3.1.2). It is presumed that the charged and thus, more polar hydrolysis products are unlikely to traverse the cell membrane as readily as the parent compound. Thus, for the drug to be effective, either as an antitumor agent or alternatively, as a cardioprotective agent, it must first enter the cell and then hydrolyze to an active form.

Concern over whether or not enough of the parent drug was available for passage into the cell at the critical time of the cells reproductive cycle resulted in an experiment to determine whether or not replenishment of ICRF-187 on a daily basis had any effect on cells different from a single drugging over the same time frame.

The rationale for this concern was that from the termination of one cell cycle to the commencement of the next was approximately 12 hours. Colony forming assays with synchronized cells that were pulsed with ICRF-159 (the racemate of ICRF-187) at different stages of the cell cycle, showed that the G_2/M border of mitosis was the critical stage in the cell cycle (Creighton *et al.*, 1979) If the drug were added at the correct time in the cells' reproductive cycles *i.e.* G_2/M phases, a great deal of the drug would be able to penetrate the cells. If it were added immediately after the critical G_2/M phase, then 12 hours would pass before the drug would be effective in the cell. Within 12 hours, and with a hydrolysis half time of 9.3 hours, only 40% of the parent drug remains as effectively able to penetrate and effect the cell.

To measure the effects of replenishment, the control, or non-replenished plate was drugged only at the beginning of the experiment after the initial 24 hour anchoring period, while the replenishment plates were drugged at the beginning and then twice more 24 and 48 hours after the initial drugging.

3.2.2 METHODS AND MATERIALS

For the most part, the preparation of ICRF-187 and its delivery were as described in Figure 2.3.7.2. The differences between this experiment and the single agent experiments described in previous sections is the number and volume of cells used and most importantly, the drugging procedure employed. These are described in considerable detail below and illustrated in Figures 3.2.2.1.1 A and B.

3.2.2.1 Seeding of the Microtitre Plates with Cells

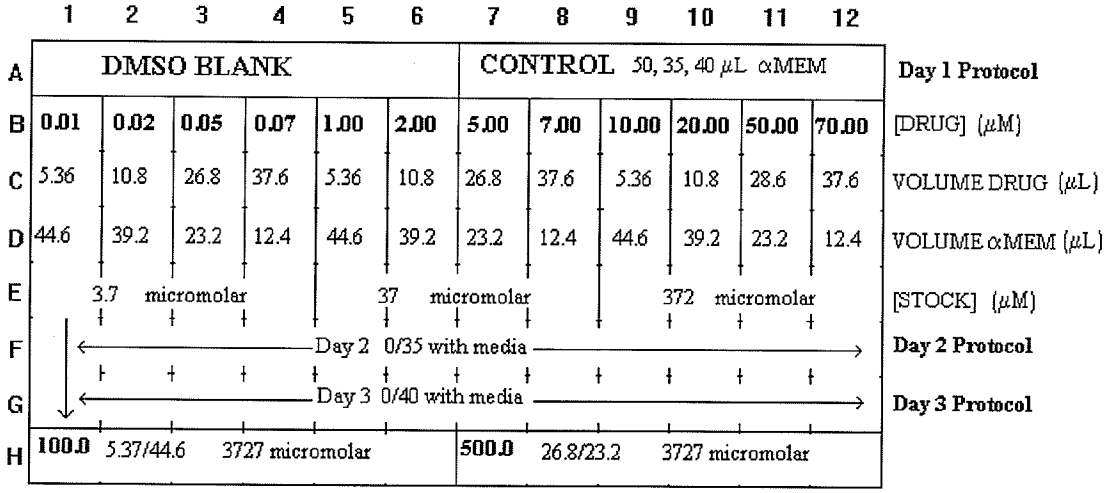
Cells were seeded into two microtitre plates in a volume of 150 μL with a count of 6667 cells mL^{-1} give 1000 cells/well. The cells were allowed to anchor for 24 hours and both plates were drugged on the following day. The drug concentration range used was from 0-500 μM. The drug was delivered in a maximum volume of 50 μL through a series of 10:1 dilutions from a stock solution of 3700 μM. The difference in volume was made up with αMEM to a total well volume of 200 μL. The plates were returned to the incubator for another 24 hours. The second drugging was performed 24 hours after the first, but drug was added only to the replenishment plate. The non-replenishment plate received only the equivalent volume of αMEM. Total volume of either drug or medium

totalled 35 μL. Since the experiment involved replenishment and not replacement of drug, the 24-hour-old ICRF-187 was not aspirated out and replaced with a fresh solution. Rather, the drug was added to the existing solution in the well and so the additional volume needed to be considered when adding the new aliquot of drug. Where the drug added initially was delivered to give a certain micromolar concentration at 200 μL, the subsequent additions were made to give the same concentration of drug at 235 μL and then for 275 μL in the final replenishment on the third day. Where the replenished experiments received 50, 35 and 40 μL of drug solutions at 24, 48, and 72 hours, the non-replenished plate received the same volumes of medium at the same times. The drug volumes and concentrations delivered to each set of wells is illustrated in Figures 3.2.2.1.1 A and B. The drugging schedule is shown in Figure 3.2.2.1.2.

The $t_{½}$ of 9.3 hours for ICRF-187 at 37°C and pH 7.4 results in only 16.7% of the original drug delivered remaining in the ICRF-187 or parental form after 24 hours. Addition of another 100% equivalent to the remaining drug results in a total drug concentration of 116.7% of the original concentrations delivered to the well on the first day. The decay of this will result in 22.8 % of the original deliver amount on the second day. And so the third drugging will give a total of 122.8% of the original drug delivered on the first day. This was computed using a decay formula

$$C = C_o \cdot e^{-K\tau}$$

NON-REPLENISHED PLATE



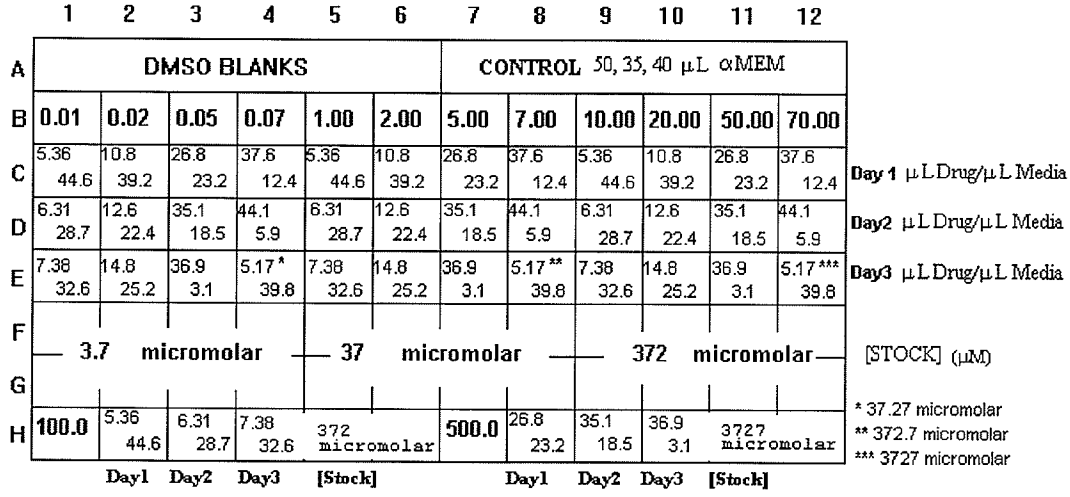
Control Wells A7-A12

Drugged Wells were arranged as shown by deliniated rows and columns above. All druggings were done in groups of six wells.

Figure 3.2.2.1.1.A ICRF-187 Non-Replenishment Drug Map

Figure 3.2.2.1.1.B ICRF-187 Replenishment Drug Map

REPLENISHED PLATE



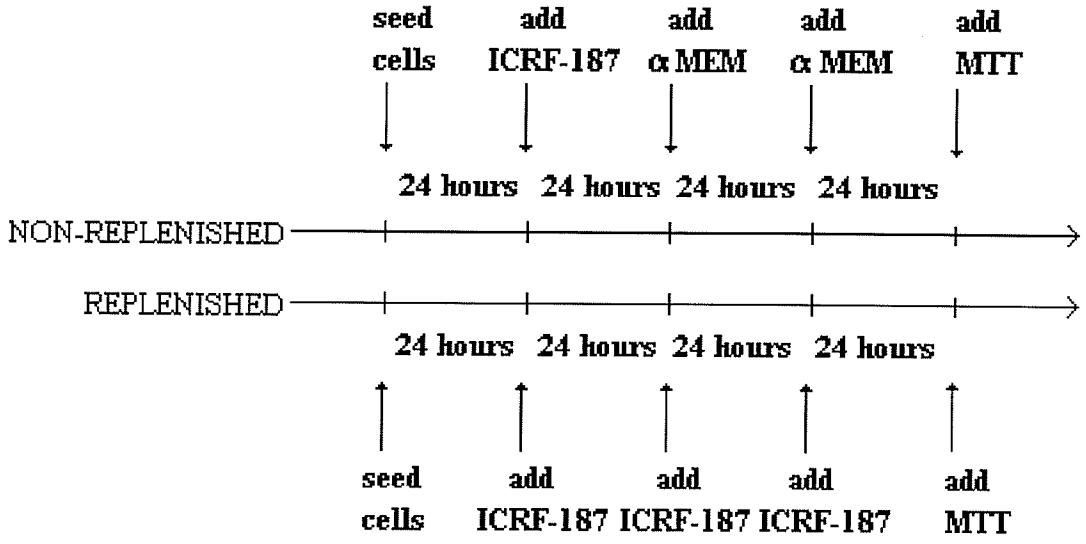


Figure 3.2.2.1.2 **Seeding And Drugging Schedule For The ICRF-187 Replenishment Experiment With CHO-AA8 Cells**

where C is the final concentration, C_o is the original concentration, τ is the time and K is the decay constant. A decay of 1 unit to 0.5 units of drug in 9.3 hours gives a decay constant of -0.074. At this rate only 16.7% of the original concentration will remain after 24 hours.

3.2.3 RESULTS

The results shown by the replenishment experiment indicated that there is little, if any significant difference in the cytotoxic effects between the replenished and non-replenished wells. There was a significance of $P < 0.001$ in the 2.0 and 5.0 μM points, but not in any other critical points in the experiment. It may be that below this concentration range there is little effect by the drug due to some minimal form of resistance. Above 5.0 μM, the cells may have been overwhelmed by the first dose and subsequent doses in the replenished plates were redundant.

The data points for both the replenished and non-replenished experiments were fitted using first a 2 parameter logistical equation and then a 4-parameter non-linear curve fitting equation,

$$Abs_{490\ obs} = (a-d) / (1+ (x/c)^b) + d$$

as described in Section 3.1.3 In the 2 parameter fits for each of the data sets, the maximum absorbance, where a was set to Abs_{490max} and d was set to Abs_{490min} The exponential value, b and c, the IC_{50} were computed. In computing the 4 parameter non-linear fit, the a and d values were set again and the b and c values computed in the 2 parameter fit were substituted in the equation as best guesses. The final b and c values were computed for both non-replenished and replenished ICRF-187 experiments.

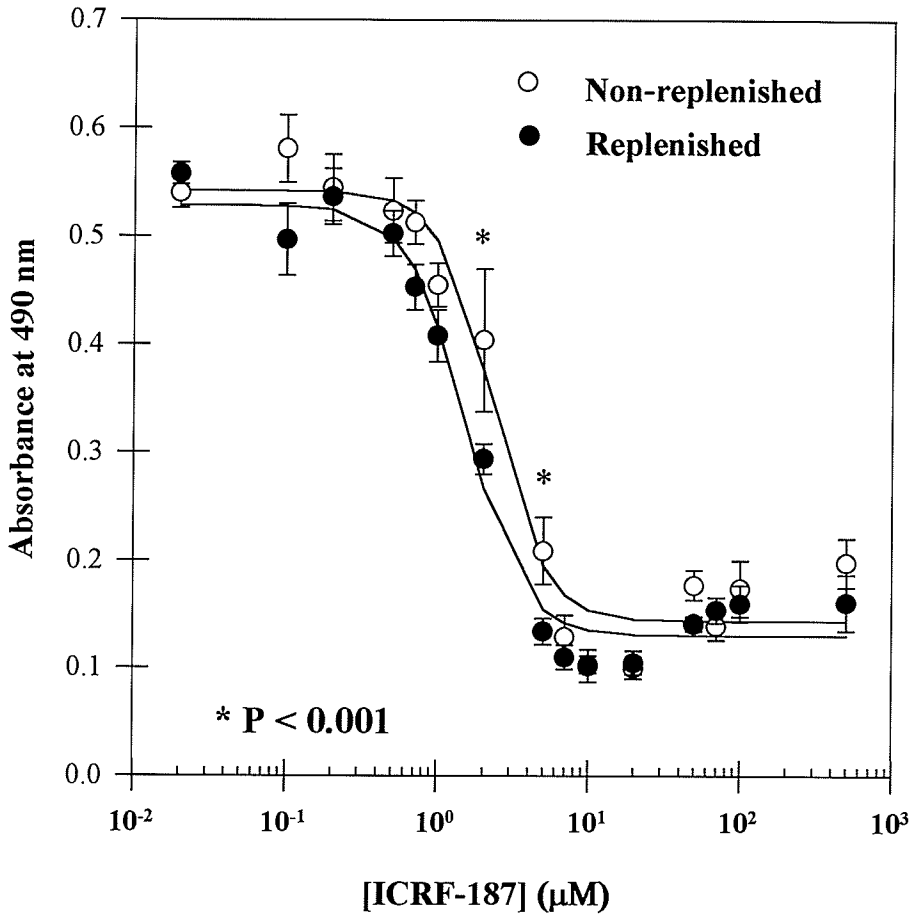


Figure 3.2.3.1 **MTT Assay Of CHO-AA8 Cell Kill After Daily Addition of αMEM (Non-replenished) or ICRF-187 (Replenished) To Wells Of Cell Culture Plates**

The comparison of the degree of damage inflicted by the range of ICRF-187 concentrations in the replenished and non-replenished assays showed that there was little difference between the results. This is especially true for the extremes of the concentration range. The 0.0-0.5 μM points were nearly identical within experimental error as were the points from 7-100 μM points. A Student's t-test indicated that only two points, the 2.0 and 5.0 μM data points were found to be significant ($P < 0.001$). There was no significant difference in the IC_{50} values between the replenished and non-replenished wells. The non-replenished IC_{50} result was 2.3 ± 0.3 μM and the replenished plate gave a result of 1.5 ± 0.2 μM. A second t-test calculation was performed using the equation below:

$$t = Mean_1 - Mean_2 / SEM_1 - SEM_2$$

The IC_{50} values for the non-replenished plate and the replenished plate are substituted into the $Mean_1$ and $Mean_2$ respectively. The corresponding Standard Errors of the Mean values are substituted into the $SEM_1 - SEM_2$ terms. For the IC_{50} values, the t value, representing the difference between the means was equal to 8.0. With 6 degrees of freedom the probability, $P < 0.001$, indicating that the points are significantly different.

3.2.4 DISCUSSION

The replenishment experiminment has shown that there is a significant difference betwenn non-replenished and replenished cells, but only in a limited area of concentrations administered over the course of 72 hours. The drug concentrations less than 2.0 μM have the same effect upon the cells whether additional drug is added or not

added. Similarely, replenishment drug doses exceeding 5.0 μM appear to have no significant difference in the toxicity observed in relation to that of the non-replenished cells upon the viability of the cells. Once the drug has affected the G_2/M phase interface, the additional doses of drug aministered to the replenishment wells appears to be redundant in the majority of doses administered and might only affect those cells that were out of synchrony (or past the critical stage of their cell cycle) with respect to the majority of the cells in the experiment. This supports the findings of Creighton *et al.*, (1979) who showed that the timing of drug delivery was critical. The initial delivery of drug was effective in arresting the cytokinetic processes within the cells. Subsequent addition of ICRF-187 was likely redundant as the interruption of the splitting had already occurred.

Only the 2.0 and 5.0 μM doses administered in a non-replenished and replenished scheme showed any significant differences ($P < 0.001$) in the toxicities mediated during the course of the experiment. However, the differences in toxicity manifested between the replenished and non-replenished plates at the 2.0 and 5.0 μM concentrations were probably due to the fact that this was a critical concentration. The IC_{50} of ICRF-187 was found to be 2.7 ± 0.2 μM. It is possible that concentrations below 2.0 were not highly effective because of a small degree of inherent degree of resistance of the cells to the drug (discussed further in Chapter 5), while a large portion of the cells was overwhelmed at drug levels above 5.0 μM. The additional cell kill seen in the 2.0 μM dose over the 5.0 μM range were potentially cells that survived one drugging in the non-replenished

experiment due to the fact they their growth was out of phase with other cells. Consequently, their mitosis event was unaffected, while the replenished plate received an additional dose of drug which was able to arrest the growth of cells not affected in the initial drugging.

However, unlike the elucidation of an IC_{50} value, where a certain concentration is able to kill 50% of a cell population, the comparison of the non-replenishment and replenishment of drugs over a specific time frame is not concerned with the amount of drug required to kill a certain percentage of cells. The IC_{50} is a critical concentration that is able to mediate a certain effect, where the comparison of the non-replenished and replenished plates is designed to show a difference between two methods of causing cell death. The range of drugs tested was 0.01-500 μM, which spans over 4.5 log units. The range that is considered significant is 2.0-5.0 μM, which is a small span within the total range of drug concentrations tested over the 72 hour course of the experiment. As a method of delivering more toxicity, the concept of replenishment is unwarranted, since a single dose slightly above 5 μM is able to achieve the same affects as several lower-concentration multiple doses over 72 hours.

Chapter 4

DUAL AGENT CYTOTOXICITY EXPERIMENTS

CHAPTER 4 DUAL AGENT CYTOTOXICITY EXPERIMENTS

4.1 INTRODUCTION

4.1.1 A Brief Introduction To Anthracyclines And Their Use In Chemotherapy

One of the most powerful groups of antitumor drugs with a host of mechanisms, some understood and others poorly understood, are the anthracycline antibiotics. These drugs are aminoglycosides of anthraquinones isolated originally from the fungus, *Streptomyces peucetius*, (Arcamone *et al.*, 1972) and are related to the antibacterial tetracyclines (Nogrady, 1988). One mechanism of antitumor activity of this class of drugs is through the intercalation of its four-membered ring system through the major groove of the DNA double helix. The anthraquinone group carries a sugar moiety ion bonded through its amino group to the phosphate backbone of the DNA. Together these perturbations stress the double helix causing single and double stranded breaks in the chain. Often, this will result in irreparable DNA damage and death of the tumor cell (Nogrady, 1988). Anthracyclines have strong activity against a wide range of malignant neoplasms, such as acute leukemia, breast cancer, Hodgkin's disease, non-Hodgkin lymphomas and sarcomas (Young *et al.*, 1981). The wide spread use of the anthracyclines as an effective chemotherapy agent is limited by the fact that they are severely cardiotoxic and doses are limited to 550 mg m^{-2} body surface area for a 21 day regimen and 700 mg m^{-2} for a weekly regimen (CPS, 1994).

The most commonly used natural anthracyclines in antitumor therapy are doxorubicin, daunorubicin, epirubicin, and idarubicin. Among these is the synthetic derivative, mitoxantrone. The basic structure of the anthracycline is shown in Figure 4.1.1.1.

The Basic Anthracycline Molecule

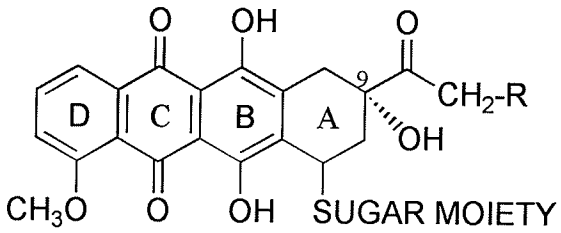


Figure 4.1.1.1

4.1.2 The Chemistry Of Anthracyclines

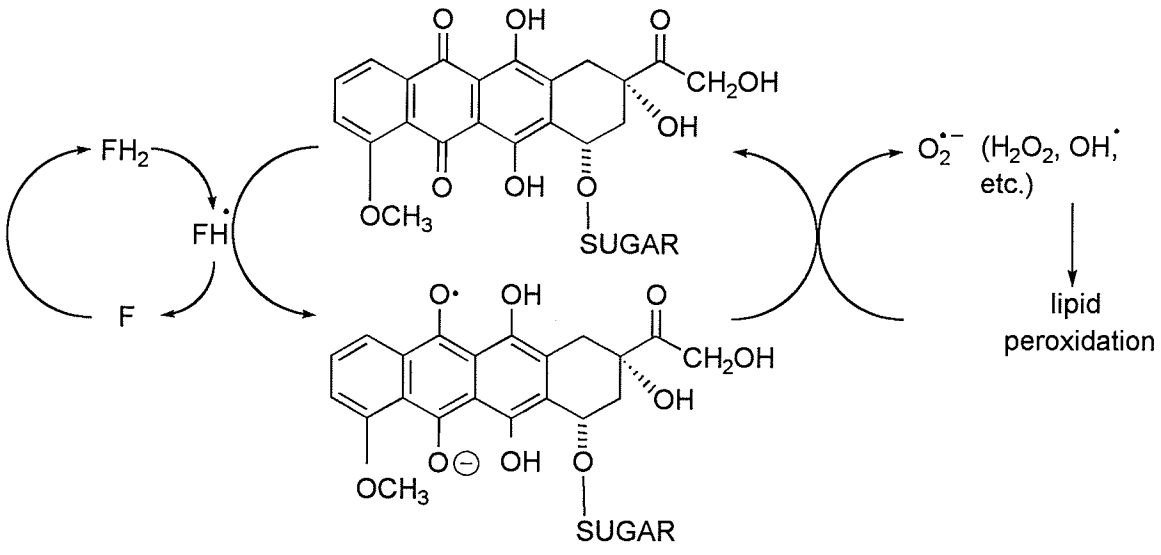
4.1.2.1 Metabolic Activation Of The Anthracyclines

Anthracyclines, and especially doxorubicin are potent antitumor compounds able to damage cells through a variety of mechanisms. It has been shown by Dalmark and Storm, (1981), that doxorubicin is able to enter cells by free diffusion. As stated earlier, the planar nature of the anthracycline molecule enables it to intercalate with DNA and affect replication by stressing the DNA helix, as well as by interfering with the spacial arrangement of the DNA, thereby sterically hindering nuclear protein function. It was shown that doxorubicin was able to induce protein-associated DNA breaks (Rowe *et al.*, 1985). Doxorubicin was shown to inhibit DNA replication and cause topoisomerase II-mediated DNA strand breakage even at low concentrations (Tewey *et al.*, 1984).

The cardiotoxicity of the anthracyclines is thought to be related to its interaction with iron and the subsequent generation of hydroxyl free radicals (Halliwell and

Gutteridge, 1985). Of particular interest are the anthracycline one and two electron reduction reactions. These reductions trigger a series of reactions generating a number of highly reactive and potentially cytotoxic species. One such reaction is the one electron reduction of doxorubicin to a semiquinone (Figure 4.1.2.1.1). According to Abella and Fisher, (1984) and Lown, (1985), the reaction can occur through the activity of a number of enzymes (*i.e.* xanthine oxidase, cytochrome P450 reductase, b5 reductase, and NADH dehydrogenase). In the subsequent reaction, the semiquinone will react rapidly with oxygen (with a rate constant of 10^8 M^{-1} sec^{-1}). In tissues, such as the heart, where superoxide dismutase levels are in low concentration, $O_2^{\bullet-}$ chemically dismutates at a

Doxorubicin One Electron Redox Cycling



(semiquinone)

Figure 4.1.2.1.1

considerably lower rate. Unable to be broken down, the hydrogen peroxide is reactive and available to partake in reactions toward a destructive end. For instance, H_2O_2 can react with a number of species to form $OH^{\bullet}$. It is this product which is available to react with a variety of biomolecules, including DNA, RNA, cell membranes and several proteins, causing widespread damage and cell death. It is for this reason that there is a great deal of attention given to the reaction mechanisms of anthracyclines, which are able to generate $OH^{\bullet}$.

Anthracyclines are also known to undergo two electron reductions to yield dihydroquinone derivatives of the parent drug. Xanthine oxidase and ferridoxin reductase are able to catalyze such two electron reductions of anthracycline compounds. The drug semiquinone is also able to react with itself to yield the parent drug and the two electron reduced parent. The rate of this reaction is very rapid (10^9 M^{-1} sec^{-1}). The hydroquinone can react with oxygen yielding hydrogen peroxide. If no oxygen is present, the reduced drug species can undergo rearrangement where the sugar is cleaved leaving a quinone methide. This species is able to alkylate both electrophiles and nucleophiles. There is some suggestion that the alkylation is a potential means of cytotoxicity (Sinha, 1980; Sinha and Gregory, 1981; Sinha *et al.*, 1984; Sinha and Sik, 1980; Ghezzi *et al.*, 1981). Figure 4.1.2.1.2 illustrates the two electron reduction and formation of quinone methide and 7-deoxyaglycone. The drug semiquinone is also known to undergo a rearrangement to yield a C-7 radical. This C-7 radical has also been proposed as a potential alkylator. The chemistry of this carbon radical is not as well understood as is that of the two

electron reduction product.

Anthracycline Two Electron Reduction And Formation Of Quinone Methide And 7-Deoxyaglycone

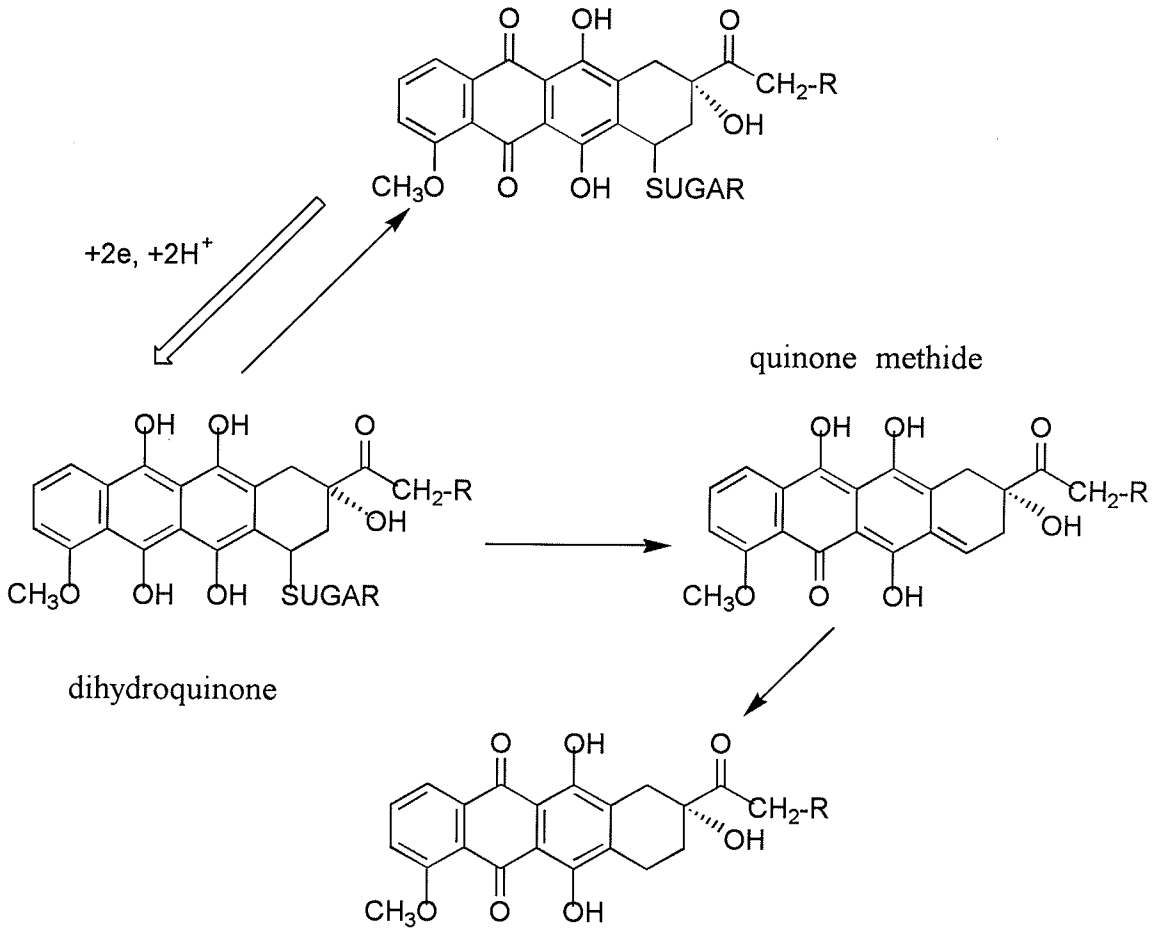


Figure 4.1.2.1.2

4.1.2.2 Oxygen Radical Dependent Cytotoxicity

There is a body of evidence which suggests that at least certain types of tumor cells can be killed by doxorubicin-induced oxygen radical formation (Tannock, 1982). Tannock (1982), has shown that doxorubicin is selectively toxic to well-oxygenated tumor cells. Electron Paramagnetic Resonance (EPR) experiments with Ehrlich tumor cells showed the presence of doxorubicin semiquinone (Sato *et al.*, 1986), while Doroshow (1986) has demonstrated the production of H_2O_2 and $O_2^{\bullet-}$ from microsomal, mitochondrial and nuclear preparations from Ehrlich tumor cells. He also showed protection of Ehrlich ascites tumor cells from 1.5 μM doxorubicin using superoxide dismutase, catalase and hydroxyl radical scavengers. Finally, Doroshow (1986), was able to measure $OH^{\bullet}$ in Ehrlich cells after exposure of the cells to doxorubicin. This was done by measuring the production of methane from DMSO.

One of the most common targets of hydroxyl free radicals is the unsaturated fatty acids in the cell membrane. Okamoto and Ogura (1985) added doxorubicin to Ehrlich cells and found that this results in peroxidation of the cell membranes and that this peroxidation could be prevented by the addition of tocopherol and enzyme Q_{10}. Thus, overall, the evidence from experiments conducted with Ehrlich cells suggests that the killing is oxygen radical mediated, which occurs after the generation of the drug semiquinone and the reduction of molecular oxygen, as described above.

Since the Ehrlich ascites are not particularly sensitive to doxorubicin and usually required drug concentration that were often greater than an order of magnitude higher than

concentrations used clinically (10^{-8} to 10^{-7} M), experiments were performed on a different line of cells. The cell line selected for these studies was the MCF-7 breast cancer cell line, which has preserved many of the basement hormonal growth factor response properties of the human breast cancer (Brooks *et al.*, 1973; Sapino *et al.*, 1986). MCF-7 is also convenient to work with since it has been grown in culture since 1974. More importantly, the versatility of the cell line allows it to transplanted back into a nude mouse resulting in the growth of a tumor undistinguishable from the initial patient biopsy. The cell line is very responsive to doxorubicin with an IC_{50} for continuous exposure of 10-30 nM, which is well within the clinical range. Breast cancer cells are probably the most important doxorubicin-responsive tumors in terms of patient numbers treated, per year (Batist *et al.*, 1986; Cowan *et al.*, 1986). Sinha *et al.*, (1987) and Sinha *et al.*, (1987) showed that doxorubicin-induced free $OH^{\bullet}$ radicals could be generated and that 60% of the radicals trapped by EPR were found to be at the cell surface. This might explain the effectiveness of superoxide dismutase and catalase in the protection of the cells. A subline of MCF-7, made resistant to doxorubicin showed only a trace amount of $OH^{\bullet}$ radicals upon exposure to doxorubicin as well as increases in expression of glutathione peroxidase and glutathione-S-transferase, which in turn allow for optimized radical clearance (Sinha *et al.*, 1987; Sinha *et al.*, 1987). Together, these studies showed that death of MCF-7 breast cancer cells exposed to doxorubicin under normal tissue culture conditions is likely a result of $OH^{\bullet}$, H_2O_2, and $O_2^{\bullet-}$.

4.1.3 The Dual Agent Cytotoxicity Experiments

Experiments described in this chapter are centred around the cytotoxic potential of a number of anthracyclines, namely, doxorubicin (DOX), daunorubicin (DNR), epirubicin (EPI), idarubicin (IDA) and the synthetic anthracycline, mitoxantrone (MIT). Tests were also conducted with bleomycin (BLM), which is not an anthracycline, but has intercalative and free radical generating capacities as do the anthracyclines (Halliwell and Gutteridge, 1985). Cytotoxicity experiments were conducted with all of these drugs in the presence and absence of the cardioprotective agent, ICRF-187 or its final hydrolysis product, ADR-925, in hopes of learning whether or not they show any protective capacity against anthracycline-induced damage on CHO-AA8 Cells. Hasinoff and Kala, (1993) have shown that ADR-925 is able to displace 100% of the transferrin-complexed iron in approximately one minute. Hasinoff, (1989 and 1990) has shown also that ICRF-187, upon hydrolysis to ADR-925, becomes a strong chelating agent, which is able to efficiently displace iron from the Fe^{3+}-(doxorubicin)$_3$ complex.

4.2 The Effect Of ADR-925 On Doxorubicin Cytotoxicity

4.2.1 INTRODUCTION

The final hydrolysis product of ICRF-187, ADR-925 has been shown to have strong iron chelating capacity. The initial intention was to determine whether or not delivery of ADR-925 to microtitre plates simultaneously with delivery of doxorubicin would prevent hydroxyl radical-mediated cytotoxicity that is known to be induced by

doxorubicin. The rationale behind the experiment was to chelate out the available iron in order to prevent it from binding the doxorubicin molecules and generating the free radicals. This was the non-preincubated ADR-925 experiment. The results of this experiment (shown and discussed below) resulted in the idea for a second experiment, where rather than adding the ADR-925 along with the doxorubicin, it was perhaps better to preincubate the cells with ADR-925 to allow for the drug to enter the cell, and chelate out the iron prior to the addition of doxorubicin. This was the preincubated ADR-925 experiment. Both experiments will be discussed throughout this section as the non-preincubated and preincubated ADR-925 experiments.

4.2.2 METHODS AND MATERIALS

4.2.2.1 Seeding Of The Cells Into Microtitre Plates

Cells were grown to near confluence in 25 cm^2 T-flasks. They were washed with Dulbecco's PBS, trypsinized, quenched and centrifuged. The pellet was resuspended in 10 mL of αMEM cell culture medium and the cells were counted. The cells were diluted to give a suspension of 10,000 cells per mL and the cells were delivered to the wells in 100 μL volumes to give final numbers of 1,000 cells per well. In the case of the non-preincubated experiment, two plates were seeded using normal cell culture medium. In the case of the preincubated experiment, the cells were resuspended in cell culture medium and counted and diluted in cell culture medium containing the appropriate concentration of ADR-925 to give three 15 mL volumes of 10,000 cells mL^{-1} with 0, 100,

or 500 μM concentrations of ADR-925. Each of these cell suspensions containing 10,000 cells per mL and different concentrations of ADR-925 was seeded into one microtitre plate per concentration of drug *i.e.*, three plates were seeded. In the case of both experiments, cells were allowed to incubate at 37°C, in darkness in an atmosphere of 5% CO_2 and 95% (v/v) air for 24 hours.

4.2.2.2 Preparation Of Drugs

ADR-925 was weighed out and dissolved 1 mg mL^{-1} in cell culture medium giving a stock solution of ~ 3,300 μM concentration. The solution was filter sterilized. This was done only on the second day in the case of non-preincubated and only on the seeding day for preincubated. In the case of the former, ADR-925 was dissolved and diluted to ten fold to 327 μM. In the case of the latter, 3,300 μM a preparation was made. No drug was added to one vial of cells, which was a control. To the second vial of cells, 457 μL of the ADR-925 solution was added to give a concentration of 100 μM. To the third vial, 2,283 μL of drug were added to give a 500 μM drug concentration. Thus, drug was added immediately during seeding for the preincubation experiment and the cells were exposed to ADR-925 for 24 hours prior to delivery of doxorubicin.

Preparation of doxorubicin for stock solution for both experiments was as described in Chapter 2. For these experiments a 1mM solution was removed from the refrigerator and allowed to warm to room temperature. Serial ten fold dilutions were made in borosilicate glass test tubes with sterile 150 mM NaCl solution to give concentrations of 1, 10, and 100 μM doxorubicin.

4.2.2.3 *Delivery Of Drugs To The Experiments*

Drug delivery for the non-preincubated ADR-925 experiment was as follows: The 327 μM ADR-925 solution was delivered to one microtitire plate in a volume of 42.1 μL. The total volume of the wells was to be 275 μL, and so this volume resulted in a final ADR-925 concentration of 50 μM. Each well also received 7.9 μL of αMEM, to give a total delivery volume of 50 μL. The other plate in this experiment received 50 μL of αMEM. Doxorubicin was delivered to both plates as follows: doxorubicin solution were prepared and diluted as described above, in 150 mM NaCl solution. Volumes as low as 2.75 μL and 13.75 μL were delivered to the wells to give the appropriate doxorubicin concentrations for each set of wells in a total of 275 μL. Differences in the volumes delivered were made up to 100 μL with sterile 150 mM NaCl solution and each of solutions was mixed thoroughly. The range of doxorubicin concentrations spanned in the experiment was 0.01-50.0 μM plus a control well containing no doxorubicin. The plates were allowed to incubate in the conditions described above for a period of 48 hours.

Drug delivery for the preincubated ADR-925 experiment was as follows: The 1mM doxorubicin solution, having been diluted as described above, was delivered in volumes ranging from 2.0-100 μL to giving the desired drug concentration for a total well volume of 200. Well volumes were made up to 200 μL with sterile 150 mM NaCl solution and mixed. The range of doxorubicin concentrations spanned in the experiment was 0.01-50.0 μM plus a control well containing no doxorubicin. The plates were allowed to incubate in the conditions described above for a period of 48 hours.

4.2.2.4 *End-point Detection*

The effects of the drugs on the cells were measured using MTT end-point detection. MTT solution (2.5 mg mL^{-1}) was delivered to each well in 25 μL volumes and allowed to incubate for 4 hours. After incubation, the medium was aspirated out carefully, ensuring that the cells and their dye were not disrupted. The formazan dye was dissolved by addition of 100 μL of spectral grade DMSO and the plates were read on a Thermomax plate reader at λ = 490 minus λ =650 nm.

4.2.3 RESULTS

4.2.3.1 *The Non-preincubated ADR-925 Experiment*

The cytotoxicity plots for this experiment are illustrated in Figure 4.3.2.1.1. Examination of these plots suggests that there is little difference between survival of plates which contained 50 μM ADR-925 and those which contained none. The data points for both the 0 μM and 50 μM ADR-925 experiments were fitted using first a 2 parameter logistical equation and then a 4-parameter non-linear curve fitting equation,

$$Abs_{490\,obs} = (a-d) / (1+ (x/c)^b) + d$$

as described in Section 3.1.3 In the 2 parameter fits for each of the data sets, the maximum absorbance, where a was set to Abs_{490max} and d was set to Abs_{490min} the exponential value b and the IC_{50} c were computed. In computing the 4 parameter non-linear fit, the a and d values were set again and the b and c values computed in the 2 parameter fit were substituted in the equation as best guesses. The final b and c values

were computed for both non-replenished and replenished ICRF-187 experiments. The comparison of the degree of damage inflicted by the range of doxorubicin concentrations in the 0 μM and 50 μM ADR-925 assays showed that there was a difference between the results. Computation of IC_{50} values showed that the plate containing no ADR-925 had a doxorubicin-mediated IC_{50} of 0.50 ± 0.14 μM and the plate containing 50 μM ADR-925 had an IC_{50} value of 0.55 ± 0.20 μM.

4.2.3.2 The Preincubated ADR-925 Experiment

The doxorubicin cytotoxicity profiles of the 0, 100, and 500 μM ADR-925 assays are illustrated in Figure 4.2.3.2.1. They show some difference in the zero doxorubicin concentrations in that there are differences in the amount of cell growth seen among the plates. The most growth is seen in the plates where there is no ADR-925 and the least is seen in the plate containing 500 μM ADR-925. Calculation of the doxorubicin IC_{50} values for the three assays was done as in the non-preincubated experiment. The only difference was that for all of the 4 parameter fits, the $d = Abs_{490min}$ values were constrained to 0, making all of the computations 3 parameter fits Table 4.2.3.2.1 displays the IC_{50} and exponential factors for the calculations for all three assays.

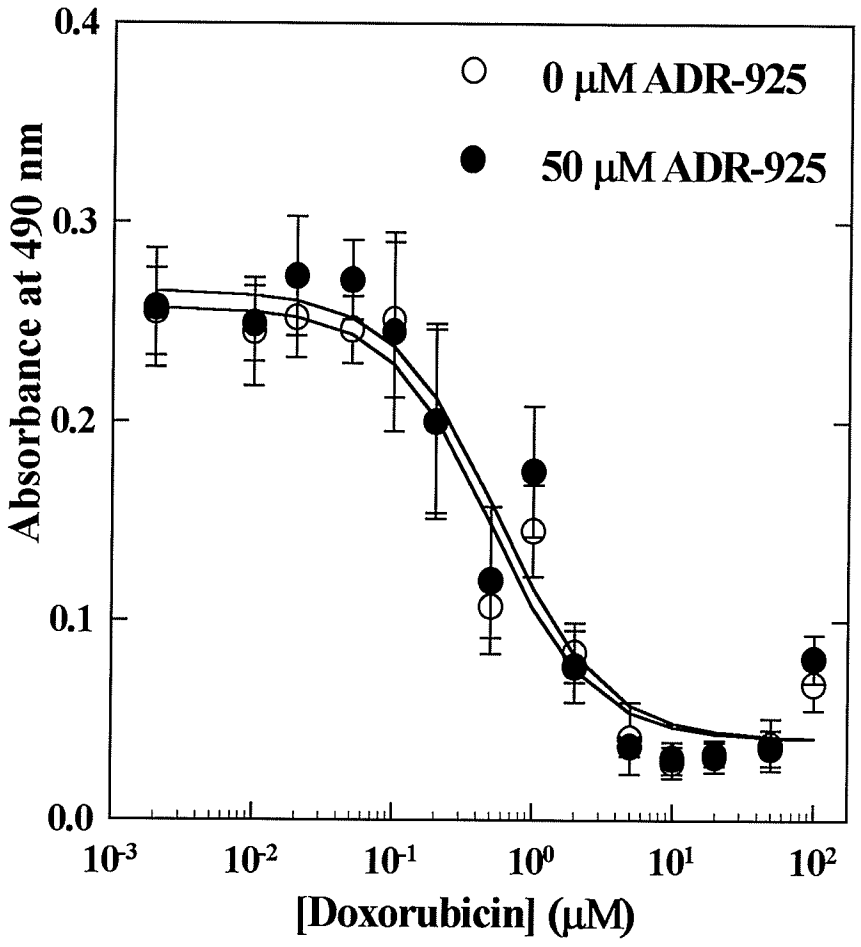


Figure 4.2.3.1.1 **MTT Assay Of Doxorubicin-mediated Cytotoxicity In The Presence And Absence Of 50 μM ADR-925 Incubated Simultaneously For 48 Hours With CHO-AA8 Cells.**

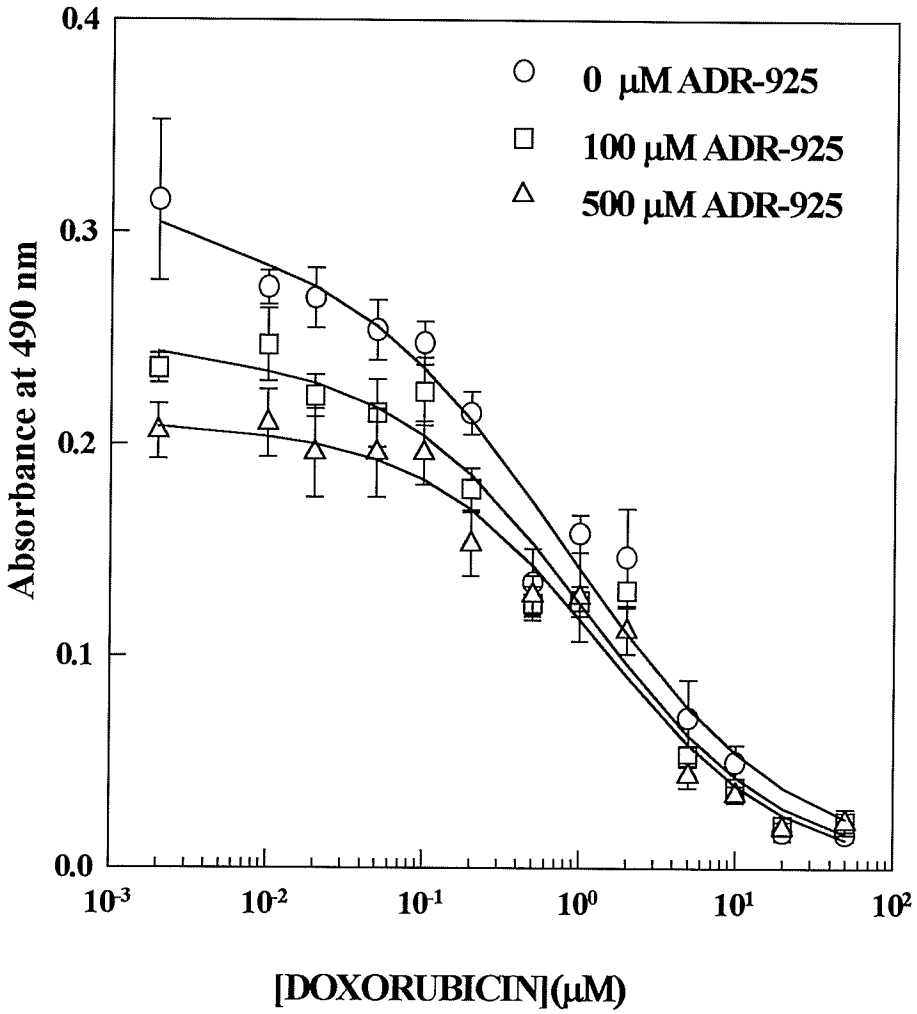


Figure 4.2.3.1.2 **MTT Assay Of Doxorubicin-mediated Cytotoxicity In The Presence Of 0, 100, and 500 μM ADR-925 Incubated With CHO-AA8 Cells For 24 Hours Prior To A 48 Hour Incubation With A Range Of Doxorubicin Concentrations.**

Table 4.2.3.2.1

50% Inhibitory Concentrations And Exponential Factors For Doxorubicin-mediated Cytotoxicity In The Presence Of 0, 100, and 500 μM ADR-925 Incubated For 24 Hours Prior To 48 Hour Incubation Of Doxorubicin With CHO-AA8 Cells.

[ADR-925] (μM)	IC_{50} (μM)	SEM	Exponential Factor	SEM
0*	0.79	0.24	0.60	0.08
100*	1.08	0.30	0.24	0.01
500*	1.42	0.30	0.75	0.10

* All $d = _{Abs490min}$ value were set to 0 for a 3 parameter fit.

4.2.4 DISCUSSION:

The two doxorubicin cytotoxicity experiments conducted with different concentrations and incubation times of ADR-925 show that there is a difference in the cytotoxicity mediated by doxorubicin when the ADR-925 is administered. The delivery of ADR-925 at the same time as doxorubicin was delivered was of little consequence in damaging or protecting the cells. The IC_{50} value for the plate containing no ADR-925 had a doxorubicin-mediated IC_{50} of 0.50 ± 0.14 μM and the plate containing 50 μM ADR-925 had an IC_{50} value of 0.55 ± 0.20 μM. There was no significant difference between the two values or essentially between the two curves. The results of the non-preincubated experiment prompted the idea that perhaps two changes were needed in the experiment. The first change was based on the simple notion that perhaps in order to be of help or

harm to the cells, the ADR-925 may require more time to enter the cells (assuming it could enter at all) due to its negative charges. Once in the cells, it would chelate the iron out of the cell and its iron-carrying proteins and prevent doxorubicin-mediated free radical generation. The second change was based on the information provided by Gibco BRL through personal communication with their technical staff. The amount of exogenous iron added to the calf serum was 221 μM and upon 10:1 dilution into cell culture medium, was 22 μM. There was no specific data regarding endogenous iron in the cell or iron bound to transferrin or ferritin in the cell. Addition of 50 μM ADR-925 may not have been sufficient to have any effect on the cells, especially since it could probably not enter them. It was thought that perhaps addition of significantly higher concentrations of ADR-925 would be able to create a gradient that might increase the potential for passage of more ADR-925 into the cells. Perhaps 24 hours of preincubation would also facilitate passage of more ADR-925 into the cells, where it could alter the effect of the doxorubicin. Also, there was a 10 fold increase in the concentration of ADR-925 added in the preincubation experiment over the non-preincubated experiment. This is likely to result in changes to the cytotoxic potential of doxorubicin.

The results of the preincubation experiment showed that there was a difference in the cytotoxicity manifested by the doxorubicin when the cells were allowed to preincubate with ADR-925 and at higher concentrations. In conclusion, it can be stated that the preincubation of cells with different doses of ADR-925 24 hours prior to delivery of doxorubicin shows a protective effect since addition of 100 μM ADR-925 increased the

IC_{50} value to doxorubicin by roughly 37% above the control, and addition of 500 μM increased the IC_{50} value by 79% over the control.

There were some differences observed in the 0 μM doxorubicin wells, where the only differences among the wells was the concentration of ADR-925. The average absorbances of the 0, 100 and 500 μM wells were 0.315, 0.236, and 0.206 absorbance units, indicating that there is either a cytotoxic effect within the ADR-925 at increasing concentrations or that there is at least an inhibition of growth. The difference among these absorbances is large enough and the standard deviations sufficiently small that the differences would not be due to a random seeding error.

4.3 The Cytotoxicity Of ICRF-187 In The Presence And Absence Of Constant Concentrations Of Doxorubicin Incubated For 48 Hours With CHO-AA8 Cells

4.3.1 INTRODUCTION

The purpose of this experiment was to measure the cytotoxicity of ICRF-187 over a range of 0-500 μM where 0, 0.2 and 0.4 μM doxorubicin were held constant over the range of ICRF-187 tested. The experiment was set up to detect any additive or synergistic effects of doxorubicin in combination with ICRF-187. A comparison of IC_{50} values for the ICRF-187 was conducted for the cytotoxicity experiments to compare the effects of doxorubicin on the survival of the cells.

The concentrations were selected based on a preliminary experiment where the IC_{50} was computed to be 0.25 μM. Based on these data the 0.2 and 0.4 μM doxorubicin

concentrations were selected for the experiments. The rationale in using of these concentrations was that they would provide enough toxicity to the cells in the experiment, but not overwhelm them. Thus, if any additional cytotoxicity was manifested, this would be detected. Also, if there was any additional toxicity mediated by the addition of the doxorubicin, this too could be measured. The levels which were selected, *i.e.* 0.2 and 0.4 μM represent IC_{25} and IC_{65} values, respectively.

4.3.2 METHODS AND MATERIALS

4.3.2.1 Seeding Of Cells Into Microtitre Plates

CHO-AA8 cells were grown and seeded as previously described in 4.2.2.1. Three microtitre plates were seeded for this experiment. The cells were allowed to incubate for 24 hours prior to the delivery of drug.

4.3.2.2 Preparation Of Drug

ICRF-187 was prepared by dissolving 1 mg mL^{-1} in αMEM, filter sterilizing with a 0.2 μm syringe filter and diluted serially diluting by 10:1 to 37.3, 3.7, and 0.37 μM solutions. Doxorubicin was prepared from a 100 μM stock solution by 10:1 dilution to 10 μM and then preparing 1.0 and 2.0 μM solutions from the 10 μM stock. Approximately 5 mL of each solution was required in order to deliver 50 μL of solution per well per plate. Dilutions of the doxorubicin were made in sterile 150 mM NaCl solution into borosilicate glass test tubes.

4.3.2.3 *Delivery Of The Drugs To The Microtitre Plates*

Sterile 150 mM NaCl was delivered to one of the plates and this plate served as a 0 μM doxorubicin control plate. Doxorubicin was delivered to the remaining plates in 50 μL volumes per well. The initial concentrations of the doxorubicin solutions delivered to the plates was 1.0 and 2.0 μM and in a total well volume of 250 μL, this will be diluted to the required concentrations of 0.2 and 0.4 μM. The delivery of ICRF-187 was made through additions of the drug to the wells in volumes no less than 6.7 and no greater than 33.5 μL and made up to a total of 50 μL with αMEM. After the addition of the drugs, the plates were allowed to incubate for 48 hours in the growth chamber in conditions described in section 4.3.2.

4.3.2.4 *End-point Detection*

The effects of the drugs on the cells was measured using MTT end-point detection. MTT solution (2.5 mg mL^{-1}) was delivered to each well in 25 μL volumes and allowed to incubate for 4 hours, after which the medium was aspirated out, ensuring that the cells and their dye were not disrupted. The formazan dye was dissolved by addition of 100 μL of spectral grade DMSO to each well. The plates were read on a Thermomax plate reader at λ = 490 minus λ =650 nm.

4.3.3 RESULTS

The IC_{50} values and the exponential values computed from the equation.

$$Abs_{obs\ 490} = (a-d) / (1+ (x/c)^b) + d$$

and the parameters are as described in 4.2.3. The calculations were made using 4 parameter fits in all cases. The results of the cytotoxicity experiments are summarized in Table 4.3.3.1. The cytotoxicity profiles of the experiments are illustrated in Figure 4.3.3.1.

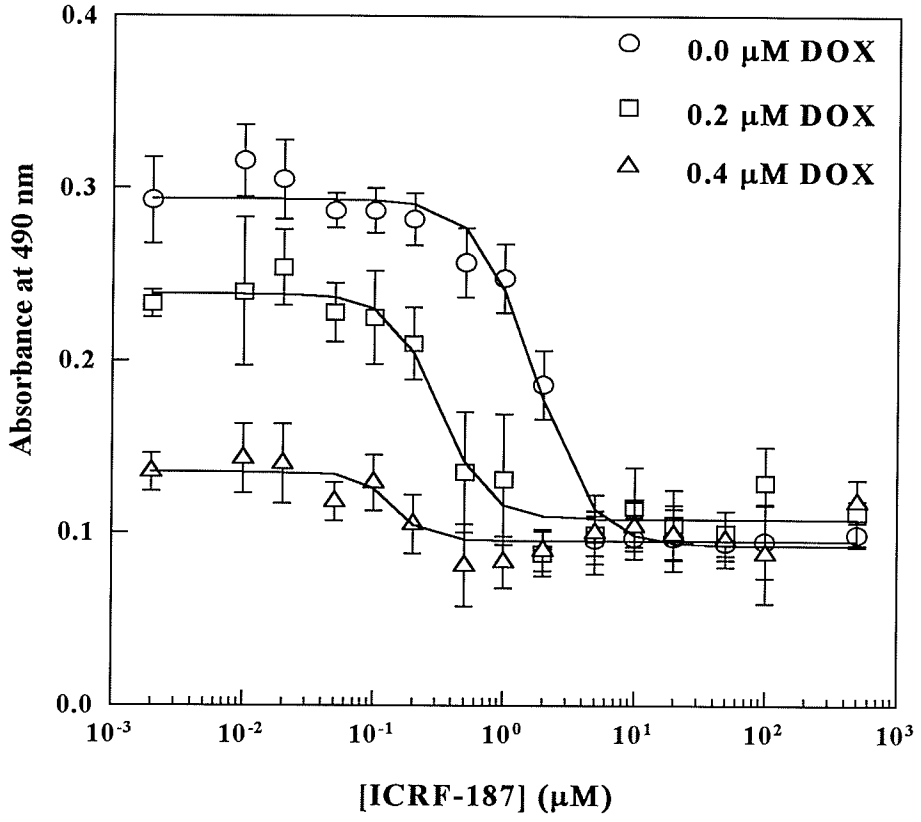


Figure 4.3.3.1 **MTT Assay Of ICRF-187-mediated Cytotoxicity In The Presence Of 0, 0.2, and 0.4 μM Doxorubicin Incubated For 48 Hours With CHO-AA8 Cells.**

Table 4.3.3.1

50% Inhibitory Concentrations And Exponential Factors For ICRF-187-mediated Cytotoxicity In The Presence Of 0.0, 0.2, and 0.4 μM Doxorubicin Incubated For 48 Hours With CHO-AA8 Cells.

[DOX] (μM)	IC_{50} (μM)	SEM	Exponential Factor	SEM
0.0	1.7	0.2	2.0	0.4
0.2	0.31	0.05	2.3	0.7
0.4	0.14	0.05	3.4	0.3

4.3.4 DISCUSSION

As is clearly seen in Figure 4.3.3.1 and verified by the calculations of IC_{50} values, there are substantial differences in the cytotoxic effects of ICRF-187 in the presence and absence of doxorubicin. The plate containing ICRF-187 without doxorubicin showed an IC_{50} value of 1.7 ± 0.2 μM, while those plates containing 0.2 and 0.4 μM doxorubicin gave IC_{50} values of 0.31 ± 0.05 μM and 0.14 ± 0.05 μM, respectively. These numbers are indicative of substantial changes in the cytotoxic potential of ICRF-187. The addition of 0.2 μM doxorubicin results in a 82% decrease from the plate containing ICRF-187 alone, in the ICRF-187-mediated IC_{50}, while the plate containing 0.4 μM doxorubicin showed a 92% decrease in the amount of ICRF-187 required to kill 50% of the cells.

These results must be viewed with a critical approach since the results are not what they appear. Though the numbers indicate the enhancement of toxicity by doxorubicin, there is a point at which the doxorubicin alone becomes the mediator of toxicity. It will be shown in the next section that the cytotoxicity of doxorubicin is approximately 10 fold higher than that of ICRF-187. The IC_{50} for doxorubicin is roughly 0.2 μM and the curve for the 0.2 μM doxorubicin reflects the contribution of ICRF-187 to the cytotoxicity mediated against the cells. However, the cell death in the 0.4 μM doxorubicin plot is likely due largely to the doxorubicin itself. The curve reflects that there is only a small overall change in the absorbance values as the concentration of ICRF-187 increases as compared with the other curves on the graph.

4.4 The Cytotoxicity Of Anthracyclines And Other Chemotherapy Drugs In The Presence And Absence Of Constant Concentrations Of ICRF-187 Incubated For 48 Hours With CHO-AA8 Cells

4.4.1 INTRODUCTION

The purpose of these experiments was to measure the cytotoxicity of a range of group of anthracyclines as well as bleomycin while measuring any additive or synergistic cytotoxicity or protective capacity with a constant concentration of ICRF-187. In the case of doxorubicin, ICRF-187 concentrations were not only held constant at 0 and 1.5 μM, but an additional experiment was conducted at 2.0 μM as well. The selection of 1.5 and 2.0 μM represent IC_{25} and IC_{40} values, respectively. The experiments were conducted with

a range of concentrations for doxorubicin, daunorubicin, epirubicin, idarubicin, mitoxantrone, and bleomycin and each was conducted in the presence and absence of 1.5 μM ICRF-187 (except doxorubicin, as discussed above).

4.4.2 METHODS AND MATERIALS

4.4.2.1 Seeding Of Cells Into Microtitre Plates

In all cases except in the case of the doxorubicin experiment, two plates of CHO-AA8 cells were required for each set of drug experiments. One plate measured the cytotoxicity of the drug in question, and the other measured the effect of the drug in the presence of 1.5 μM ICRF-187. Cells were grown in the conditions described earlier in this chapter. They were seeded 1,000 cells per well by delivering 100 μL of cell suspension carrying 10,000 cells mL^{-1}. They were allowed to incubate without drug for 24 hours in the growth chamber and were drugged following the 24 hour anchoring period.

4.4.2.2 Preparation Of Drugs

All of the drugs tested were readily soluble in aqueous solutions. The anthracyclines were all dissolved in 150 mM NaCl and diluted to make appropriate substock solutions in 150 mM NaCl as well. These drugs were extremely toxic antibiotics and were not filter sterilized since for the most part they required no sterilization, but most importantly, they bind to all surfaces and drug is lost from solution. All dilutions were made in disposable borosilicate glass test tubes. Bleomycin was dissolved in αMEM

and dilutions were made in αMEM. The drug was filter sterilized and all dilutions were made in disposable sterile 6 mL polyethylene test tubes.

Preparation of ICRF-187 was made by weighing out a very small quantity of ICRF-187 (~0.1mg) and dissolving it in cell culture medium to make 6.0 μM ICRF-187. The 6.0 μM solution was filter sterilized through a syringe filter of 0.2 μm porosity. Sufficient volumes were prepared to deliver 50 μL per well. This was approximately 10 mL per experiment (*i.e.* two plates) In a total well volume of 200 μL the delivery of a 6.0 μM solution made the final ICRF-187 concentration 1.5 μM.

4.4.2.3 Delivery Of Drugs To The Microtitre Plates

After the 24 hour anchoring, the anthracycline drugs and bleomycin were added to the plates first. The ranges of drug used were different in almost each case, since each drug was found to have a characteristic cytotoxicity profile. In the case of each drug, preliminary cytotoxicity experiments were conducted to reveal the correct range wherein the IC_{50} could be found. In each case the profile was refined increments of drug concentration change were altered to isolate the IC_{50} value more accurately. Certain portions of a given cytotoxicity profile required smaller increments of drug concentration change to fully reveal all of the points of inflection within the curve. In the case of doxorubicin, the final drug range used was 0.01-5.0 μM in one experiment and 0.01-200.0 μM in another. Daunorubicin was drugged from 0.01-50.0 μM. Epirubicin was delivered using a range from 0.01-50.0 μM. Idarubicin was approximately 10 fold more cytotoxic

than the other anthracyclines and so the drug concentrations tested were from 0.001-5.0 μM. Mitoxantrone was delivered in a concentration range spanning 0.01-50.0 μM. Bleomycin was delivered in a range of 0.01-200 μM. In the case of all drugs, all volumes delivered to the wells were no less than 10 and no greater than 50 μL. Differences in volume were made up to 50 μL with 150 mM NaCl or αMEM in the case of bleomycin. The 6.0 μM ICRF-187 was delivered last and each well was thoroughly mixed. The plates were allowed to incubate for a period of 48 hours in the growth chamber.

4.4.2.4 End-point Detection

The results of the cytotoxicity experiments were measured using the MTT assay. After the 48 hour incubation with the drugs, 20 μL of 2.5 mg mL^{-1} MTT solution was added to each well and the plates were allowed to incubate for another 4 hours. After development of the formazan dye, the medium was aspirated out of each well and the formazan crystals were dissolved in 100 μL spectral grade DMSO. After the crystals were thoroughly dissolved, the results of the assay were read on the Thermomax plate reader at $\lambda = 490$ minus $\lambda = 650$ nm.

With respect to the MTT assay of the mitoxantrone experiment, it was shown that in the higher ranges of mitoxantrone concentration, the mitoxantrone would adhere to the microtitre plate walls and upon delivery of DMSO, the mitoxantrone would dissolve and give unusually low absorbance readings. The results from the 20 and 50 μM wells plummeted well below zero absorbance. This is unusual since the absorbance due to

anthracyclines such as doxorubicin tend to be higher than they should be, usually by 0.005 absorbance units for both 20 and 50 μM doxorubicin solutions. This was due to the detection of these drugs by the 650 nm reference beam.

An experiment was conducted, where mitoxantrone was delivered in 20 and 50 μM concentrations to six wells each in a total volume of 200 μL per well. The drug was allowed to incubate for 48 hours as is customary for a cytotoxicity experiment, except that there were no cells. After 48 hours, the wells were aspirated. No MTT was added. A volume of 100 μL of DMSO was added to each well and the absorbances were read. The average absorbance value for the 20 μM mitoxantrone wells was -0.043 ± 0.002 and the average for the 50 μM wells was -0.091 ± 0.016. Thus, after reading the MTT assay for mitoxantrone, these values were subtracted from the actual reading to give a corrected value.

4.4.3 RESULTS

The plots in this section illustrate the cytotoxic potential of doxorubicin (Figures 4.4.3.1 and 4.4.3.2), daunorubicin (Figure 4.4.3.3), epirubicin (Figure 4.4.3.4), idarubicin (Figure 4.3.4.5), mitoxantrone (Figure 4.4.3.6), and bleomycin (Figure 4.4.3.7) incubated for 48 hours with CHO-AA8 cells in the presence and absence of 1.5 μM ICRF-187. Table 4.4.3.1 shows the exponential factors and IC_{50} value for the above mentioned drugs, both in the presence and absence of 1.5 μM ICRF-187 and Figure 4.4.3.8 illustrates the structures of the different anthracyclines tested.

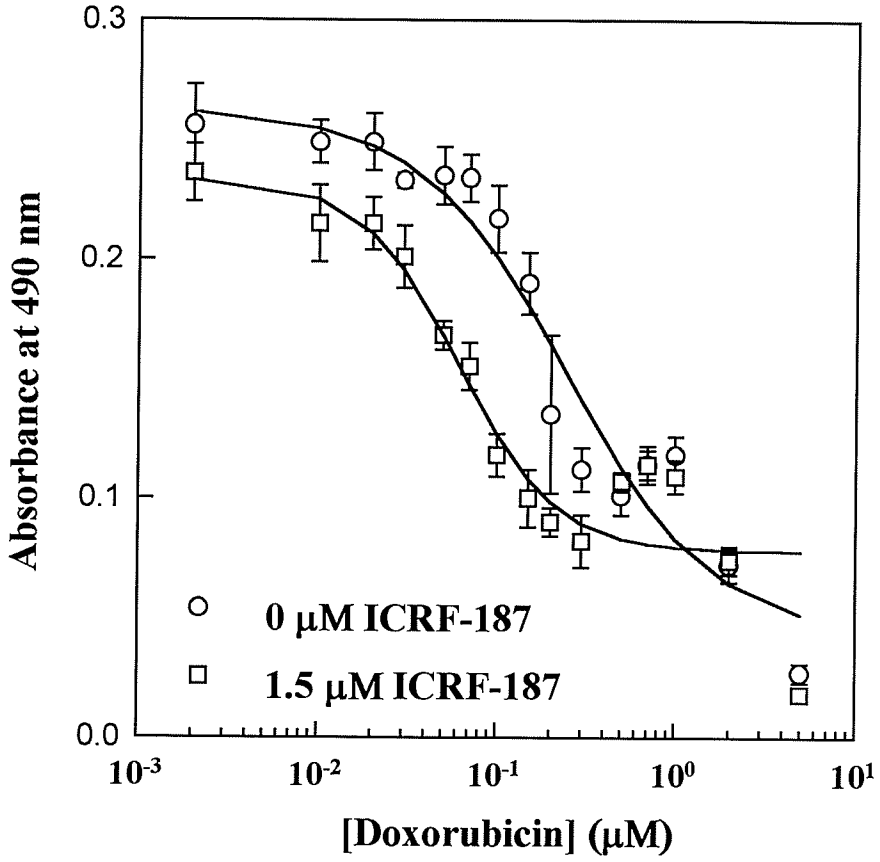


Figure 4.4.3.1. **MTT Assay Of Doxorubicin-mediated Cytotoxicity In The Presence And Absence Of 1.5 μM ICRF-187 Incubated For 48 Hours With CHO-AA8 Cells.**

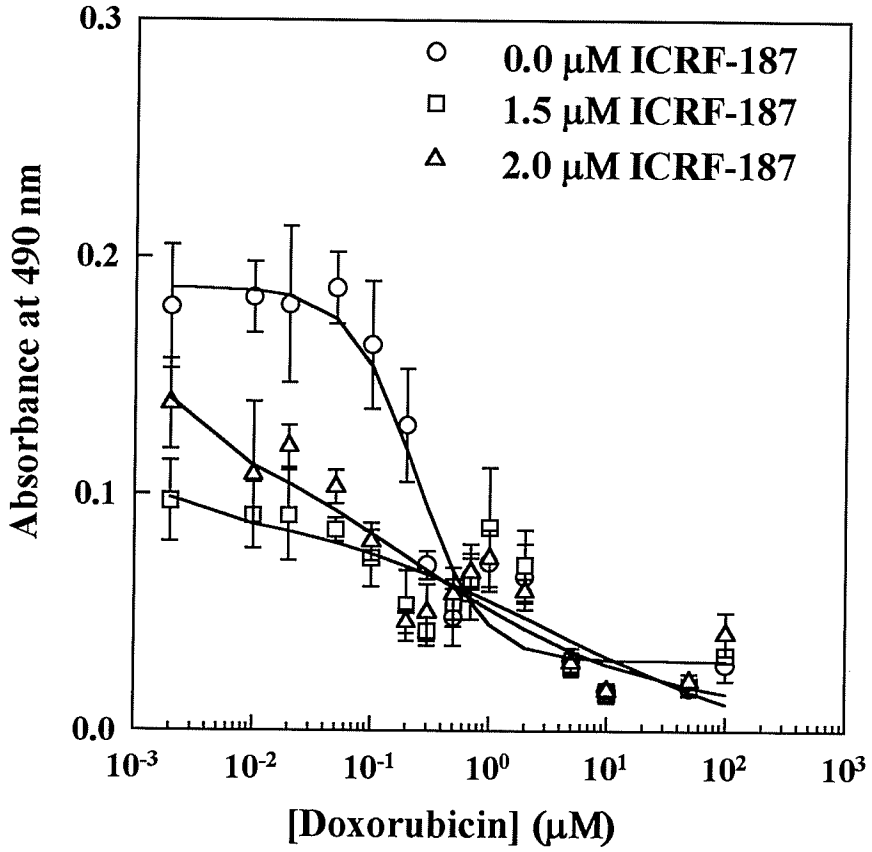


Figure 4.4.3.2. **MTT Assay Of Doxorubicin-mediated Cytotoxicity In The Presence Of 0, 1.5, And 2.0 μM ICRF-187 Incubated For 48 Hours With CHO-AA8 Cells.**

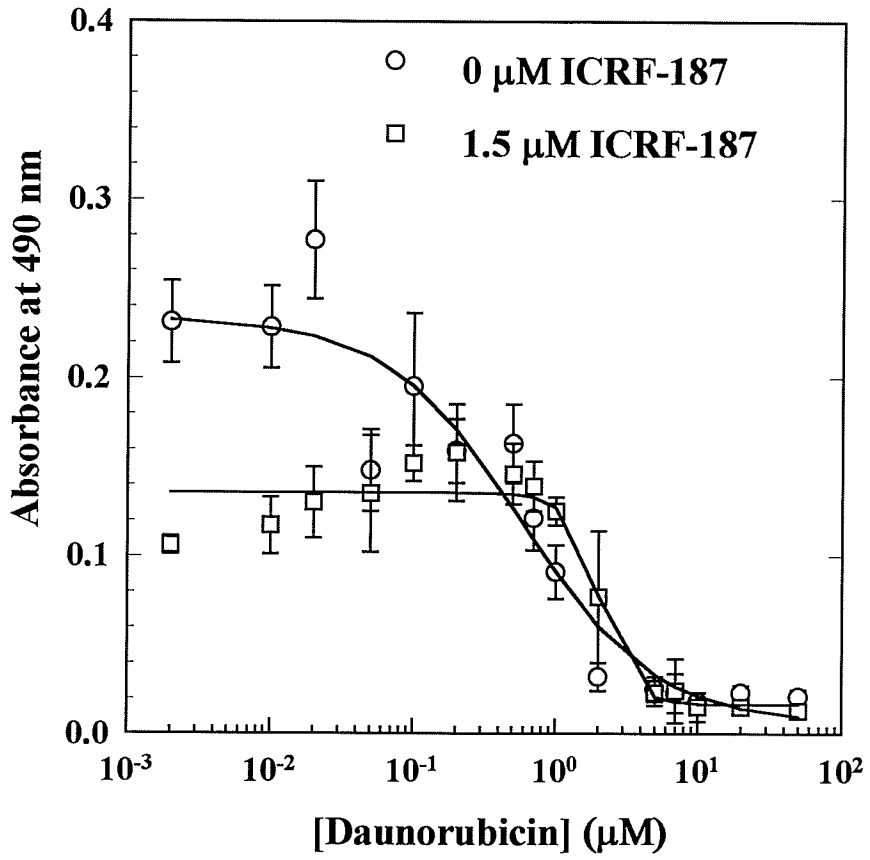


Figure 4.4.3.3. **MTT Assay Of Daunorubicin-mediated Cytotoxicity In The Presence And Absence Of 1.5 μM ICRF-187 Incubated For 48 Hours With CHO-AA8 Cells.**

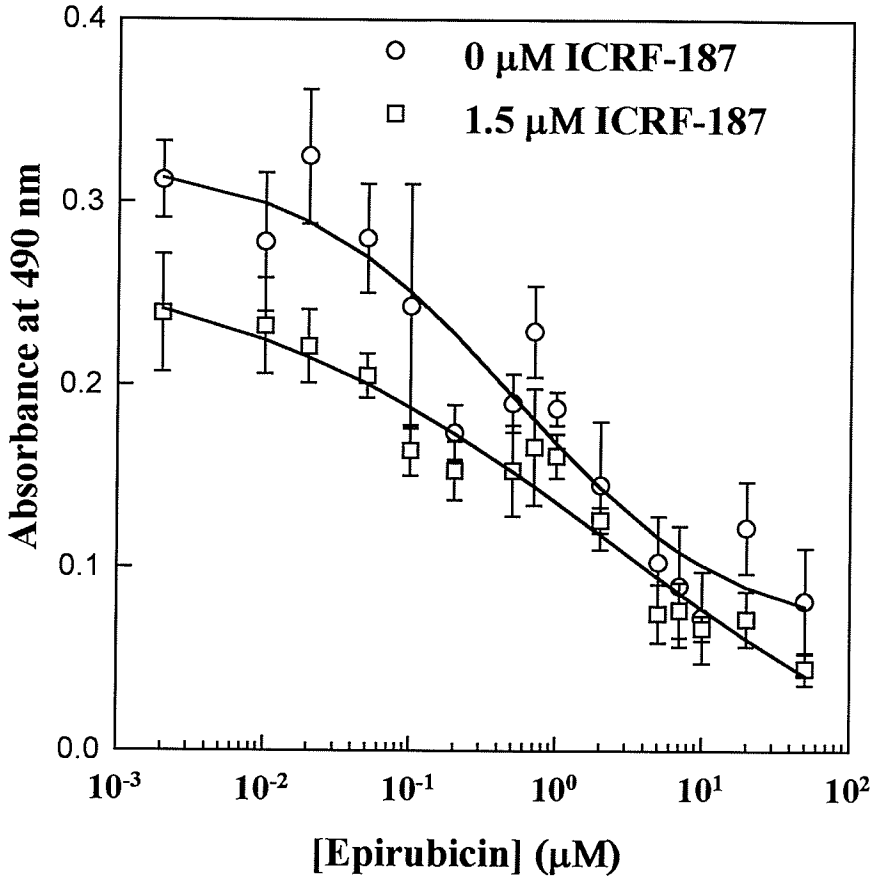


Figure 4.4.3.4. **MTT Assay Of Epirubicin-mediated Cytotoxicity In The Presence And Absence Of 1.5 μM ICRF-187 Incubated For 48 Hours With CHO-AA8 Cells.**

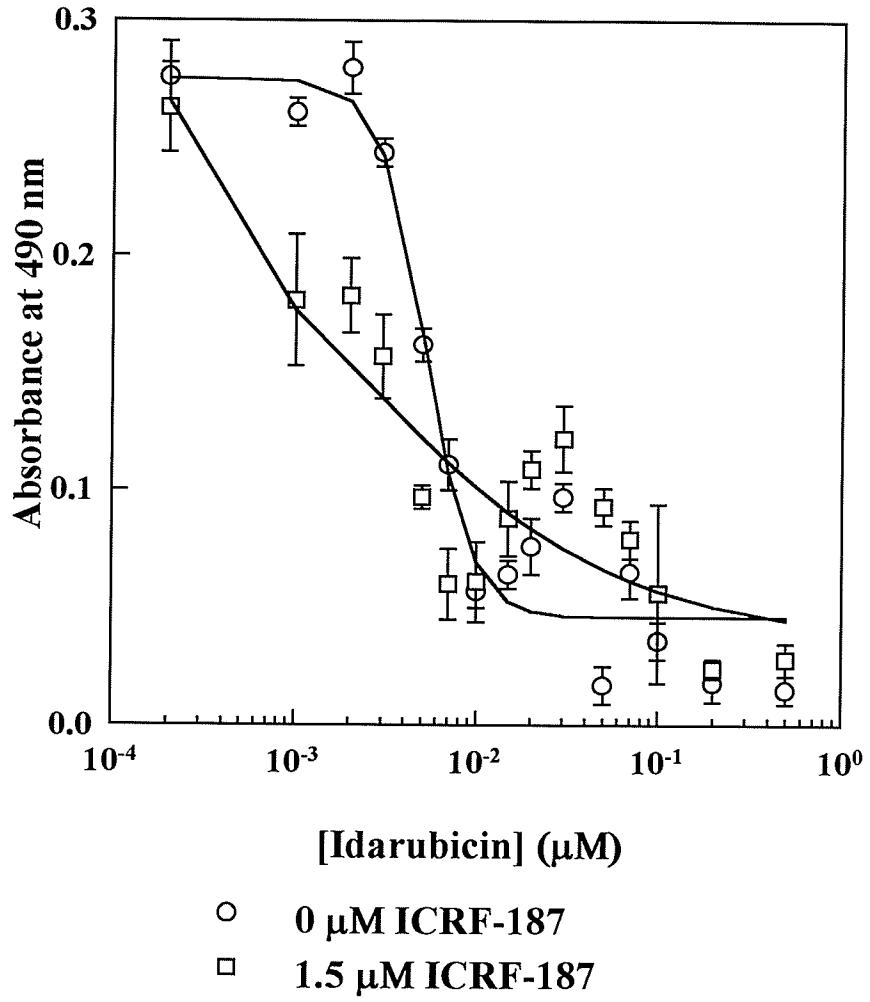


Figure 4.4.3.5. **MTT Assay Of Idarubicin-mediated Cytotoxicity In The Presence And Absence Of 1.5 μM ICRF-187 Incubated For 48 Hours With CHO-AA8 Cells.**

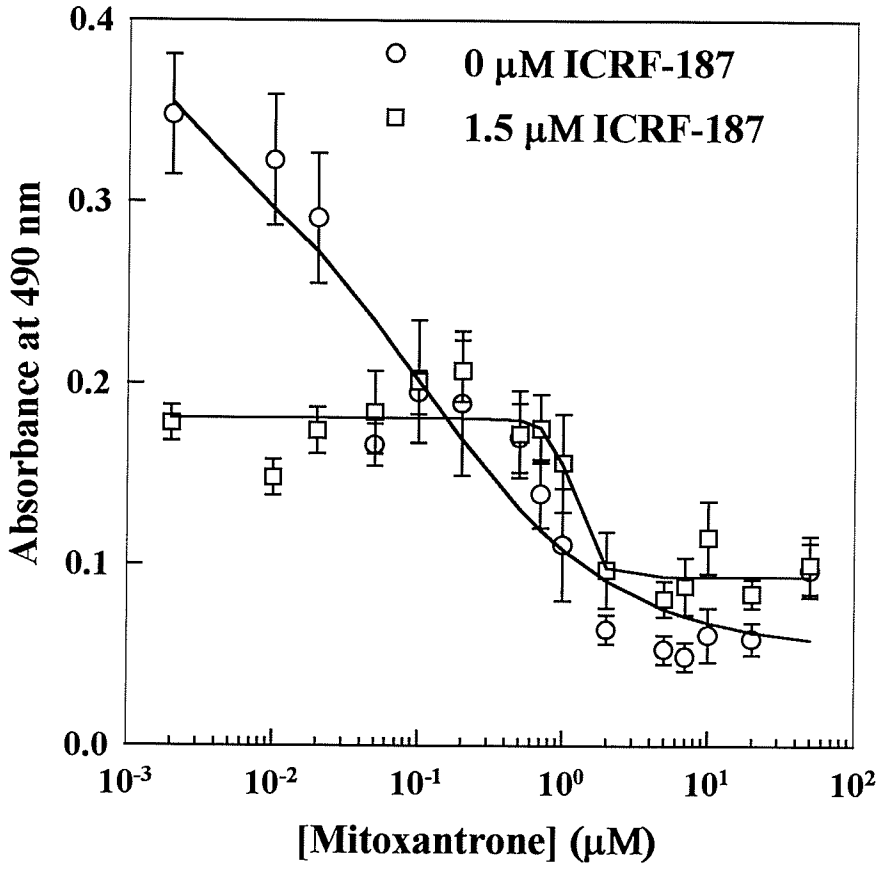


Figure 4.4.3.6. **MTT Assay Of Mitoxantrone-mediated Cytotoxicity In The Presence And Absence Of 1.5 μM ICRF-187 Incubated For 48 Hours With CHO-AA8 Cells.**

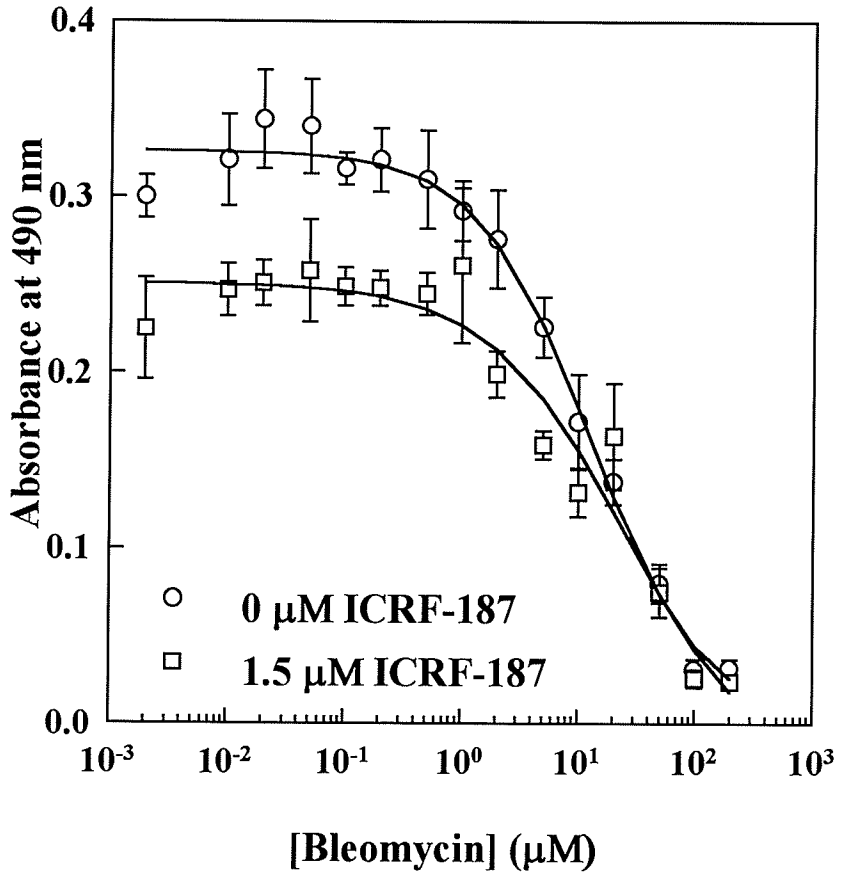
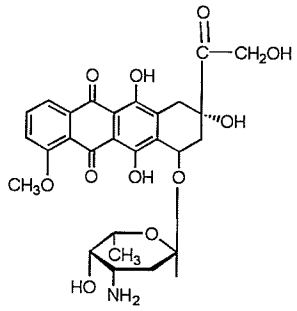


Figure 4.4.3.7. **MTT Assay Of Bleomycin-mediated Cytotoxicity In The Presence And Absence Of 1.5 μM ICRF-187 Incubated For 48 Hours With CHO-AA8 Cells.**

Figure 4.4.3.8

The Anthracyclines

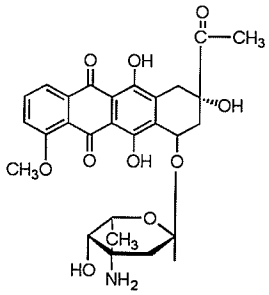


DOXORUBICIN·HCl

Molar Mass: 579.99 g/mol

Molar Absorbtivity :12,200 $M^{-1}cm^{-1}$

Solubility in ddH_2O: >10 mM

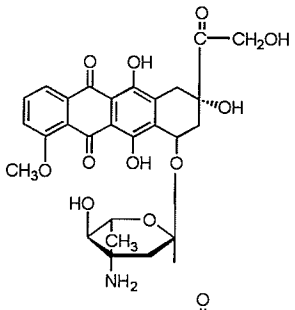


DAUNORUBICIN·HCl

Molar Mass: 563.97 g/mol

Molar Absorbtivity :12,100 $M^{-1}cm^{-1}$

Solubility in ddH_2O: >10 mM

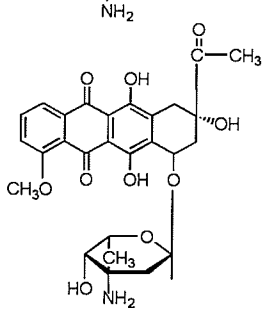


EPIRUBICIN·HCl

Molar Mass: 579.99 g/mol

Molar Absorbtivity :12,200 $M^{-1}cm^{-1}$

Solubility in ddH_2O: >10 mM

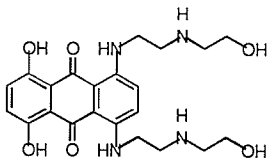


IDARUBICIN·HCl

Molar Mass: 533.9 2 g/mol

Molar Absorbtivity :NA

Solubility in ddH_2O: >10 mM



MITOXANTRONE

Molar Mass: 412.5 g/mol

Molar Absorbtivity : NA

Solubility in ddH_20: > 10 mM

Table 4.4.3.1 50% Inhibitory Concentrations And Exponential Factors For Cytotoxicity Experiments With Drugs Incubated With CHO-AA8 Cells For 48 Hours In The Presence And Absence Of ICRF-187.

Drug Combination	IC_{50} (μM)	SEM	Exponential Factor	SEM
DOX + 0.0 μM ICRF-187*	0.35	0.12	0.81	0.20
DOX + 1.5 μM ICRF-187*	0.17	0.19	0.37	0.13
DOX + 2.0 μM ICRF-187*	0.26	0.22	0.34	0.08
DOX + 0 μM ICRF-187*	0.39	0.09	0.78	0.13
DOX + 1.5 μM ICRF-187*	0.19	0.09	0.51	0.11
DNR + 0 μM ICRF-187*	0.64	0.22	0.86	0.22
DNR + 1.5 μM ICRF-187*	2.6	0.5	2.1	0.5
EPI + 0 μM ICRF-187*	1.5	0.8	0.42	0.08
EPI + 1.5 μM ICRF-187*	1.8	0.7	0.45	0.06
IDA + 0 μM ICRF-187*	0.006	0.002	1.15	0.27
IDA + 1.5 μM ICRF-187*	0.004	0.003	0.40	0.10
MIT + 0 μM ICRF-187*	0.19	0.11	0.43	0.08
MIT + 1.5 μM ICRF-187*	1.31	0.89	0.48	0.22
BLM + 0 μM ICRF-187*	12.6	1.2	0.89	0.07
BLM+ 1.5 μM ICRF-187*	17.8	4.2	0.85	0.58

* $d = Abs\ 490_{min}$ constrained to 0.

The IC_{50} values and the exponential values computed from the equation.

$$Abs_{490\ obs} = (a-d) / (1+ (x/c)^b) + d$$

and the parameters are as described in 4.2.3. The calculations were made using 3 parameter fits in all cases.

4.4.4 DISCUSSION

The cytotoxicity experiments with anthracyclines and bleomycin in the absence of ICRF-187 show the following degrees of cytotoxicity:

Idarubicin > Mitoxantrone > Doxorubicin > Daunorubicin > Epirubicin > Bleomycin.

In the case of idarubicin, the cytotoxicity did not appear to be appreciably reduced by the addition of 1.5 μM ICRF-187. The differences between the IC_{50} values in the absence and presence of ICRF-187 were 0.006 ± 0.002 and 0.004 ± 0.003, respectively. The values are similar enough and the SEM values large enough that they render the differences as insignificant. The differences between the mitoxantrone experiments in the absence and presence of ICRF-187 are substantial, though the error in the case of the ICRF-187-free experiment is high. There is a 6.9 fold increase in the IC_{50} value when ICRF-187 is added to the experimental conditions. The errors in the values are substantial, however it does not change the fact that there is a difference between the two cytotoxicity profiles. doxorubicin is cytotoxicity is doubled in the presence of 1.5 μM ICRF-187. In the case of daunorubicin, the cytotoxicity appears to be reduced in the presence of ICRF-187. Roughly four times as much daunorubicin was required to kill the same number of cells

in the presence of ICRF-187 as was required in its absence. In the case of epirubicin, there was little significant change in the cytotoxicity mediated by the drug when ICRF-187 was added. The cytotoxicity mediated by the bleomycin was also not altered significantly by the addition of ICRF-187.

The increases in cytotoxicity manifested by doxorubicin in the presence of ICRF-187 is in agreement with experiments performed by Wadler *et al.*, (1987), who showed that ICRF-87 was able to add to the cytotoxicity of the doxorubicin in experiments with S180 murine sarcoma cells. On the contrary, daunorubicin cytotoxicity appears to be reduced in the presence of ICRF-187. This is also expected, since this effect was seen in cytotoxicity experiments conducted with OC-NYH cells (Sehested *et al.*, 1993). Sehested suggests that the differences seen in the protective effects exhibited by the ICRF-187 on daunorubicin and doxorubicin is related to the difference in the membrane transport and cellular pharmacokinetics of the two anthracyclines. This is reasonable since the uptake of anthracyclines is passive and only the efflux is active (Le Bot *et al.*, 1988). Cytotoxicity experiments with rat liver epithelial cells and human hepatocytes showed that the sensitivity of cells is influenced by a number of additional factors: The activity or functional stability of cells, the compound and its metabolites or degradation products will all influence the cytotoxic potential of a drug (Del Tacca *et al.*, 1985; Beijnen, *et al.*, 1986). Hence, the differences in the cytotoxicities. Since drug cytotoxicities among cell types differ (Le Bot *et al.*, 1988), it is not possible to speculate freely on the trends manifested in the cytotoxicity of anthracyclines on CHO-AA8 cells. Similarly, the

protective or cytotoxic potential of ICRF-187 is not readily apparent for the same reasons.

The doxorubicin experiment with 0, 1.5, and 2.0 μM ICRF-187 show reasonable agreement with the results of the doxorubicin ± 1.5 μM ICRF-187 in terms of IC_{50} values and general cytotoxicity. The value for the 2.0 μM ICRF-187 experiment shows an increase in the IC_{50} where a decrease would be expected, however, the error is substantial in all of these experiments and so the change in the trend is not to be considered significant.

The errors in the calculations are substantial in the case of anthracycline experiments. Examination of all of the plots will show that all of them manifest an increase in the absorbance (*i.e.*, the survival of the cells) between a point soon after the cytotoxic concentrations are indicated on a cytotoxicity plot. This is true for all of the anthracyclines, regardless of the range at which they are cytotoxic. Initially, it was thought that this was due to a dilution problem, however, alteration of the dilutions and overlapping dilution experiments showed that this was not a dilution problem, rather it was a characteristic of anthracyclines. This phenomenon can be explained by the finding that topoisomerase II can be inhibited by doxorubicin concentrations which exceed a critical value of between 0.5 and 2.5 μM (Tewey *et al.*, 1984). Increased concentrations of doxorubicin are known to inhibit the induction of protein-associated DNA strand breaks (Potmesil *et al.*, 1983), which is then expected to inhibit its own cytotoxicity. Survival of cells was found to level off at higher doxorubicin concentrations according to Barranco, (1984). This was found to be true to only to a degree since increases in doxorubicin

concentrations above 2.5 μM were shown to increase the amount of doxorubicin-mediated cell kill. Different mechanisms, such as hydroxyl free radical generation by doxorubicin-iron complexes will form in greater quantity at higher doxorubicin concentrations and increase cell kill. Though, these phenomena are described for doxorubicin, it seems likely that they occur to varying degrees for all of the anthracyclines.

The unusual inflections seen in the anthracycline cytotoxicity profiles is due, possibly to this above mentioned strand break inhibition. In calculating the IC_{50} values, these inflections cause difficulties, since the errors in both the exponential values and the IC_{50} values become unavoidably large. This is remedied to a degree by constraining the minimum absorbance value to zero.

4.5 Cytotoxicity Of Doxorubicin With IC_{25} Concentrations Of ICRF-154 And ICRF-161

4.5.1 INTRODUCTION

Cytotoxicity experiments were conducted to measure the cytotoxicity of doxorubicin in the presence and absence of ICRF-154 and ICRF-161. The purpose of these experiments was to determine whether or not there was any protective or additional cytotoxic effects manifested by the addition of an IC_{25} concentration by either of the two bisdioxopiperazines which were added to a range of doxorubicin concentrations. ICRF-154 and ICRF-161 were chosen for these experiments since they were among the more toxic drugs readily available.

4.5.2 METHODS AND MATERIALS

The cell seeding, drug preparations and delivery were all performed as described in section 4.4. The IC_{25} concentration were calculated based on the IC_{50} values calculated for the these compounds in Chapter 3. The IC_{25} of ICRF-154 is 8.0 μM and the IC_{25} for ICRF-161 is 12 μM. These were delivered in 50 μL volumes of cell culture media to a total of 200 μL. The drugs were prepared as concentrated stocks; the ICRF-154 was prepared as a 32 μM solution and the ICRF-161 was prepared as a 48 μM solution. Doxorubicin was serially diluted and delivered as described in the previous section. All incubations and end-point detection was conducted as previously described.

4.5.3 RESULTS

Figure 4.5.3.1. illustrates the cytotoxicity of doxorubicin in the presence and absence of ICRF-154 and Figure 4.5.3.2 illustrates the cytotoxicity profile of doxorubicin in the presence and absence of ICRF-161. Table 4.5.3.1 compares the IC_{50} values and the exponential factors from these plots.

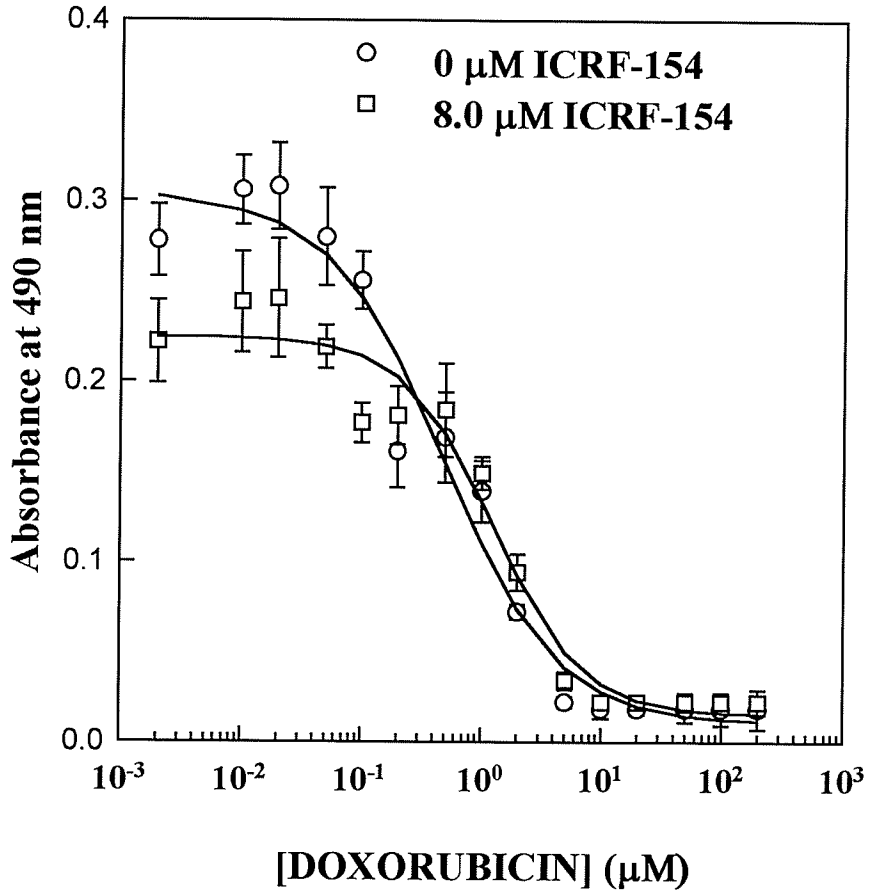


Figure 4.5.3.1 **MTT Assay For Doxorubicin-mediated Cytotoxicity In The Presence And Absence Of 8.0 μM ICRF-154 Incubated For 48 Hours With CHO-AA8 Cells.**

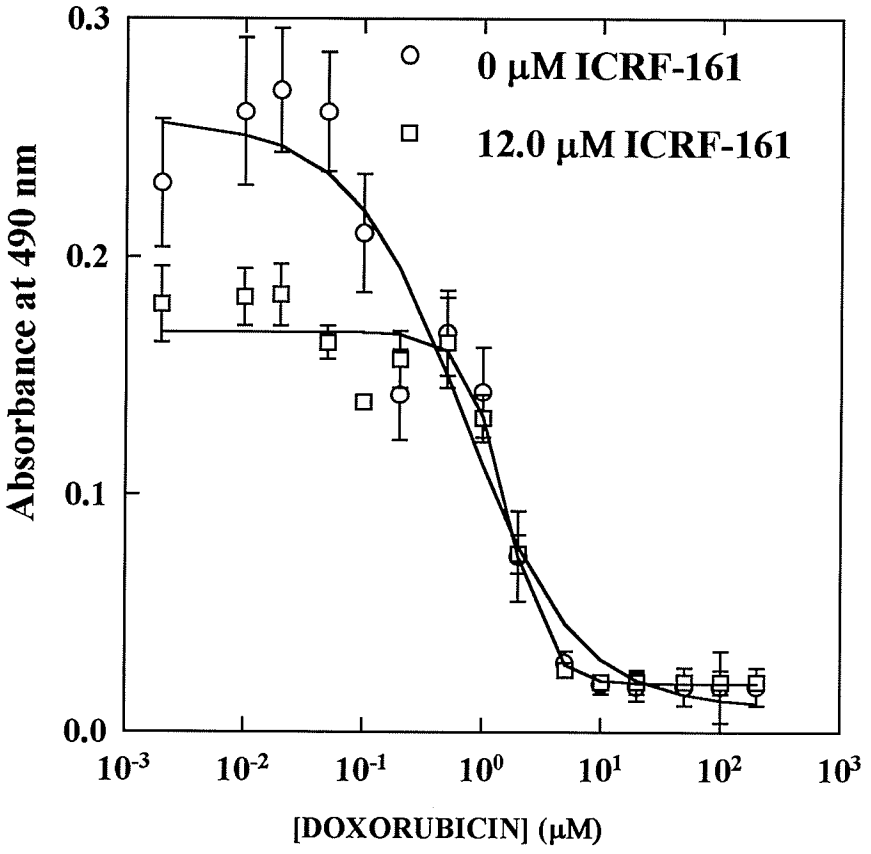


Figure 4.5.3.2 **MTT Assay For Doxorubicin-mediated Cytotoxicity In The Presence And Absence Of 12.0 μM ICRF-161 Incubated For 48 Hours With CHO-AA8 Cells.**

Table 4.5.3.1 50% Inhibition Concentrations And Exponential Factors For Cytotoxicity Experiments With CHO-AA8 Cells Incubated For 48 Hours With Doxorubicin And In The Presence Or Absence Of IC_{25} Concentrations Of Either ICRF-154 Or ICRF-161.

Drug Combinations	IC_{50} (μM)	SEM	Exponential Factor	SEM
DOX + 0 μM ICRF-154*	0.54	0.13	0.83	0.14
DOX + 8.0 μM ICRF-154* (IC_{25})	1.4	0.3	0.90	0.16
DOX + 0 μM ICRF-161*	0.76	0.24	0.82	0.17
DOX + 12.0 μM ICRF-161* (IC_{25})	1.9	0.3	1.4	0.33

The IC_{50} values and exponential factor were calculated using the formula shown in 4.4.3.1

* $d = Abs\ 490_{\min}$ was constrained to 0.

4.5.4 DISCUSSION

The IC_{50} values computed for the cytotoxicity experiment involving doxorubicin ± IC_{25} concentrations of ICRF-154 show that the cytotoxicity of the doxorubicin is altered in the presence of the ICRF-154. The doxorubicin IC_{50} values in the absence of ICRF-154 are within the error range of the doxorubicin values shown in section 4.4. The addition of ICRF-154 protects the cells to some degree since the doxorubicin concentration required to kill 50% of the cells is roughly 2.6 times higher than the amount required when no ICRF-154 is added. In the case of ICRF-161, the results are similar. The addition

of an IC_{25} concentration of ICRF-161 protects the cells to a degree where roughly 2.5 the amount of doxorubicin is required to kill 50% of the cells as compared to the amount required when no ICRF-161 is added.

This is different from what was seen using IC_{25} concentrations of ICRF-187, where the addition of the drug resulted in enhanced cytotoxicity and halving of the amount of doxorubicin required.

Chapter 5

THE ICRF-187-RESISTANT CELL LINE, DZR3

CHAPTER 5 THE ICRF-187-RESISTANT CELL LINE, DZR3

5.1 INTRODUCTION

5.1.1 The Enzymes Targeted In Antitumor Therapy

The rationale in using drugs for the selection of a drug-resistant line of cells is borne of the need to understand the disease process where patients undergoing chemotherapy to combat tumors have remarkable remissions only to suffer a fatal relapse from the same disease process. At the onset of the relapse, many of the patients show a resistance to a wide number of structurally unrelated antitumor drugs, hence the term multidrug resistance (MDR). Patients with other types of tumors such as those arising in the colon, kidneys, liver, and lungs tend to respond poorly to the antitumor drugs currently available. The MDR phenomenon includes cross-resistance among a broad variety of agents such as the anthracyclines (doxorubicin, daunorubicin, etc.), epipodophyllotoxins (etoposide), the vinca alkaloids (vinblastine), as well as other compounds. Experiments conducted *in vitro* with cell culture models show that this type of resistance is associated with the over-expression of the *mdr1* gene, which codes for the expression of a surface membrane protein known as P-glycoprotein (P-gp). The protein acts as an efflux pump which serves to transport drugs associated with MDR out of the cell before any cytotoxic effects can be mediated. The protein is expressed in normal tissues such as in the gastrointestinal tract, the liver and the kidneys. In those tissues it is believed to serve as an excretory pathway for toxins and xenobiotic compounds. Preliminary studies have shown that P-gp is present in tumor samples of

patients with acute leukemia, multiple myeloma, lymphomas, as well as in a variety of solid tumors. Certain compounds are able to reverse the MDR. These drugs include calcium channel blockers, phenothiazines (neuroleptic), quinidine (antiarrhythmic), antimalarial agents, antiestrogenics and other steroids, and cyclosporin (immunosuppressive agent). Though many are able to reverse MDR, their use clinically in antitumor regimens is limited by their toxicities (Lum *et al.*, 1993).

Another contributor of drug resistance in antitumor therapy are the cellular drug metabolizing enzyme known as glutathione-S-transferase (GST). These enzymes were identified in the 1960's when it was found that elevations in the expression of GST enzymes was associated with acquired resistance to alkylating anticancer drugs. Since then a great deal more has been learned about this phenotype. The GST-α family of isozymes has been firmly correlated with increased resistance to alkylating antitumor agents, which are known to be substrates for GST isozymes (Dulik *et al.*, 1987). Batist *et al.*, (1986) have shown the over-expression of the GST-π in a multidrug resistant cell line, which has served to identify a potential role for this isozyme in the detoxification of free-radical-induced DNA damage caused by the quinone metabolites of doxorubicin. Observations have been made by numerous investigators that GST-π is the predominant isozyme present in human cancer tissue and serves as a putative tumor marker in certain instances of both human and rodent disease (Shea *et al.*, 1988). Nakagawa *et al.*, (1990) have shown that transfection of the GST-π gene into activated c-H-*ras*-transformed NIH-3T3 cells resulted in the ability of the cells to withstand cytotoxic insult from drugs which are either

direct or indirect substrates of the isozyme, but not from other drugs which are not direct or potentially indirect substrates of GST-π. Thus, GST isozymes are usually involved together with other gene products in the protective effects against the stresses induced by cytotoxic agents however, they are not the sole agents responsible for certain MDR phenomena.

One of the most important and interesting target enzymes of antitumor agents from the perspective of this project is the DNA winding and unwinding enzyme, topoisomerase II. As mentioned in Chapter 3, the topoisomerases are nuclear enzymes which function to resolve topological problems associated with DNA transcription. This involves the overwinding and underwinding and catenation of DNA strands. Topoisomerases, especially topoisomerase II, which is found in eukaryotic cells is responsible for relaxing both negatively and positively wound DNA supercoils, catenation, decatenation, as well as knotting and unknotting of DNA strands (D'Arpa and Liu, 1989). Of the two topoisomerases known to exist in mammalian cells, topoisomerase IIβ is expressed at low levels throughout the cell cycle, while topoisomerase IIα is expressed at increased levels during cell proliferation and is regulated by the cell cycle. Agents, such as the more potent bisdioxopiperazine compounds in the ICRF series, target the topoisomerase IIα enzyme, and are able to effect the cells progression through mitosis by interrupting the process at the G_2/M phase boundary and are able to do so without the formation of cleavable DNA-enzyme complexes (Gorbsky *et al.*, 1994). This results in multinucleated cells which are unable to complete cytokinesis, and will eventually die.

(Creighton *et al.*, 1979). At the DNA level, cytotoxicity is mediated by an increase in sister chromatid exchanges and inhibition of DNA and RNA synthesis. In general, topoisomerase II poisons are able to produce a rapid inhibition of DNA and RNA synthesis. The inhibition is not absolute; over a range of drug dosages, the cells can produce anywhere from a partial to a full complement of DNA prior to arrest of the cell in the G_2 phase. In cells which are resistant to the effects of topoisomerase II inhibiting agents, the resistance is due to the alteration of the structure of the enzyme and altered catalytic activity (D'Arpa and Liu, 1989).

Topoisomerase II poisons include a variety of agents including doxorubicin, actinomycin D, and etoposide. Their development for use as antitumor agents was based on empirical observations, however the identification of topoisomerase II as their site of action provides a rational basis for analyzing the molecular mechanisms of cytotoxicity as well as drug resistance. This will enable improvement in the design of multiple-agent therapeutic regimens.

5.1.2 The Isolation Of An ICRF-187-Resistant Cell Line And The Characterization The Nature Of Its Drug Resistance

Working with cell culture models in the laboratory, it is possible to select for cells with increased resistance to virtually any of the cytotoxic agents used in the treatment of cancer. Though, the concentrations of drug utilized in selecting these resistant clones often greatly exceed the doses used in a clinical setting, these drugging regimens can result in

clones with an extraordinary degree of resistance to the selection drug. At times these models do not accurately model the drug resistance manifested in a patient, but they have proven advantageous in detecting and defining the relevant resistance-related alterations at the cellular level. These experiments have also lead to the elucidation of a number of underlying genetic mechanisms involved in the mediation of the drug-resistant phenotype (Biedler *et al.*, 1988).

This chapter is concerned with the selection and characterization of a line of cells derived from the parental cell line CHO-AA8 and selected for resistance to the bisdioxopiperazine antitumor compound ICRF-187. The protocol describing the selection of the cell line as well as the characterizing of the cells is described below. This chapter describes in detail the selection of a drug-resistant cell line as well as the isolation of a colony of cells from a single resistant cell. The characterization of the cell line includes a description of cell morphology during the selection period, the testing for the viability of the cells after cryogenic freezing, measurement of growth rate and doubling time, as well as the duration of the resistant phenotype after growth in the absence of drug for an extended period of time. In addition to characterizing the cells, the chapter covers experiments involving cytotoxicity testing of the cells with ICRF-187 analogues as well as structurally and functionally unrelated antineoplastic agents in order to determine whether or not resistance developed against ICRF-187 has resulted in any concomitant resistance to other antitumor agents.

5.2 SELECTION AND PROPAGATION OF THE ICRF-187 RESISTANT STRAIN OF CHINESE HAMSTER OVARY CELLS, DZR3

5.2.1 Growing the Resistant Cell Line

5.2.1.1 Medium and Growth Conditions

Chinese Hamster Ovary AA8 cells were grown from frozen stocks purchased from ATCC. The cells were grown in αMEM medium prepared as described in section 2.3.1 The cells were grown in the dark at 37°C in 5% CO_2 and 95% air (v/v) in either of 25 cm^2 or 75 cm^2 canted T-flasks.

5.2.1.2 Selection of the Optimal Container For Cell Growth And Daily Drugging

The first step in beginning the selection of a drug resistant ell line was to select the container in which the cells could be grown. The ideal container would be small in order to minimize the volume of drugged medium required for cell growth. This would be important in terms of the amount of drug required on a daily basis, especially near the end of the selection process, since drug concentrations would become very high and expensive. Initial attempts to begin the resistant clone selection from the CHO-AA8 wildtype cells in small petri dishes were unsuccessful. Though economical in terms of the volume of medium and drug used, they were difficult to manipulate and sterility of the dish was always a concern since the cover was easy to remove accidentally. Also, it was difficult to maintain correct osmolarity of the medium, since the small volumes of the dish resulted in evaporation of water from the medium. This was not a problem over the short

term of required for normal cells to reach confluence, but the inhibitory effect of ICRF-187 on the cells resulted in a much longer growth period before cells reached confluence. The 24-well plate was also unsuccessful. Once the cells were confluent and required trypsinizing and reseeding, it was found that the centrifugation and resuspension resulted in a great deal of cell loss. Since the population of cells harvested from a well or from a petri dish was small, the loss of cells left little if any net gain in the number of cells grown. The best choice for the growth of cells over a duration of daily druggings was the 25 cm^2 canted T-flask. During periods of daily drugging the cell growth was slowed significantly, and so they allowed for easy manipulation of the flask for up to a week between trypsinizing without the worry of contamination. Also, since there was a great deal of cell debris during the initial days of increased drug concentration, the use of a flask was convenient for the removal of old medium (when required), washing with Dulbecco's PBS and delivery of fresh medium and drug. The best feature of the flask was that in spite of the increase of drug required for the growth of cells in a drugged environment, it allowed for the growth of a significant population of cells that was unaffected by the losses due to centrifugation and manipulation.

5.2.1.3 Drugging and Increasing of Drug Resistance

The CHO-AA8 cells were made resistant to ICRF-187 by continually subjecting the cells to increasing doses of the ICRF-187. The incremental increases in concentration of the ICRF-187 acted as a selection agent, gradually killing off populations of cells which were unable to tolerate the drug and possibly initiating a mechanism within a

certain portion of the cell population which enabled the them to withstand increasing doses of the drug. Eventually those cells capable of growing under the conditions of high drug concentration formed the majority of the population.

Recrystallized ICRF-187 was weighed out in sufficient quantity and dissolved by sonication in αMEM+10% (v/v) calf serum at a concentration of 0.01 mg mL^{-1} (37 μM), after which the drug solution was filter sterilized and diluted appropriately into the medium of the growing cell population. Initially, the delivery was to give a drug concentration of 2 μM in a total volume of 10 mL. In the later stages of the selection process, ICRF-187 was dissolved in 10 mM concentration and delivered into cell culture flasks to give final drug concentration as high as 2 mM and 4 mM in a total volume of 5 mL. The smaller volume was in order to conserve drug. Sonication of serum-containing medium will likely result in a breakdown of the serum protein, however, since the drugs are diluted into large volumes of unsonicated media, it is not a serious problem.

The next crucial step was to decide at which concentration to start the cell selection process. The initial concentration was 10 μM. This first attempt at beginning the selection process was unsuccessful since the 10 μM starting concentration killed all of the cells in the flask. The rationale behind a high starting concentration was to minimize the time spent eliminating the weaker element of the cell population and to immediately begin the isolation of the heartier cells by using a drug concentration above the IC_{50},but below the concentration of total cell kill. This was unsuccessful. A second attempt was initiated using two flasks, drugged at 2.0 and 5.0 μM, respectively. The drug solutions were added

to flasks which were approximately 50% confluent. This number of cells allowed for a large number of possible survivors during the druggings while allowing time and room for the cells to grow for a longer period of time prior to trypsinizing. Trypsinizing was found to be disruptive to the cell growth, since reseeding resulted in a lag period of at least 12 hours prior to the resumption of growth at the exponential rate. During the initial stages of selection, the growth was stationary for as much as 24 or 48 hours prior to exponential growth. Thus, the cells were allowed to grow without disruption for at least two to three days in the drugged medium before they were trypsinized.

The increase in consumption of drug that came with the use of a T-flask as opposed to petri dishes or 24-well plates was remedied by using 5 mL of medium in which to grow the cells, rather than 10 mL. This however, created the potential for more rapid accumulation of cell waste, dead cell debris, etc. The problems of accumulation of cell debris and metabolic waste, as well as the consumption and rapid depletion of nutrients in this small volume of medium was remedied by changing the medium on a daily basis, rather than every two to three days. Since drug was added every day in the initial stages of the drugging, it was convenient to remove the old medium, wash with Dulbecco's PBS and add the drug dissolved in a 5.0 mL volume of fresh medium. Also the addition of fresh drug was added daily with fresh medium, the accumulation of waste was not a problem and the use of 5 mL of medium and drug instead of the normal 10 mL halved the amount of drug and medium used in the selection of these resistant cells.

The initial 2.0 and 5.0 μM doses of ICRF-187 were added to separate flasks of

cells and subsequently, drug was added on a daily basis to ensure sustained drug concentrations since the half-life of ICRF-187 at pH 7.40 is 9.3 hours. Only the 2.0 μM flasks was maintained since the 5.0 μM dose proved toxic to all of the cells after 2 days. After growth to near-confluence in the flask, the cells were passaged and were seeded at approximately 40,000 cells mL^{-1} into two flasks. During the course of the cells' development of drug resistance, one flask was maintained at the current dose, while the other flask received double the current dose of drug. If the cells survived and grew well in the high doses of ICRF-187, these cells were allowed to grow to confluence in the presence of drug and the lower dose flask was discarded. If the higher dosed cells did not survive or showed poor growth, they were abandoned and the lower dosed flask was grown to near-confluence in the current dose and higher doses were attempted when the cells seemed more stable in their growth at the given drug concentration.

The drugging procedure was not done according to a strict predetermined schedule. Drug was added or not added on a daily basis strictly by observation of the cells after the most recent drugging. If cells appeared to be sustaining a reasonable growth rate in spite of an increase in the concentration of drug delivered, the drug concentration was maintained or even increased within one day. If the response was again good, the drug could again be increased. If the increases resulted in a large amount of cell death or simply very poor growth, drug was either maintained at levels prior to the cell death, or it was withheld completely until the cells had the appearance of recovering from the damage inflicted from the drugging. The decision to add or not add drug on a given day

was made on a daily basis seven days a week. Observation of the cells on a daily basis was essential. The course of drug additions is illustrated in Figure 5.2.1.3.1, which shows the course of selection of drug-resistant cells over the course of a day to day drugging regimen. Within the space of 110 days, the cells proceeded from surviving in 2.0 μM ICRF-187 to surviving 2.0 mM doses. Attempts to grow the cells at 4.0 mM were not successful. The index of resistance attained, which was defined as the IC_{50} value of the resistant cells divided by the IC_{50} value of the wildtype cells, was roughly 900 fold. Selection of doxorubicin-resistant H69AR cells required 14 months of selection in order to acquire a 32-fold resistance to doxorubicin (Mirski *et al.*, 1987). The selection of the ICRF-187-resistant cells seemed rapid when considering the 900-fold increase in resistance.

In order to preserve a quantity of these cells in case of unforseen problems, such as total cell death or culture contamination, the drug resistant cells were propagated, growing in 2 mM ICRF-187. When enough cells were grown to fill ten cryovials with 1 mL each containing 5×10^6 cells mL^{-1}, the cells were prepared for cryogenic storage and subsequently frozen in liquid nitrogen as described in section 2.3.8.

Not all cells were frozen, however. It was not yet known whether the selection of the resistant line had in some way resulted in a class of cells that were resistant to drugs, but perhaps vulnerable where freezing was concerned. Or perhaps, the drug induced acquisition of drug resistance mechanisms had produced cells unable to withstand freezing. For this reason, a flask of the resistant cells was maintained until it was shown

that the frozen cells were able to recover from cryogenic storage and resume normal growth. This is discussed in more detail in section 5.4.

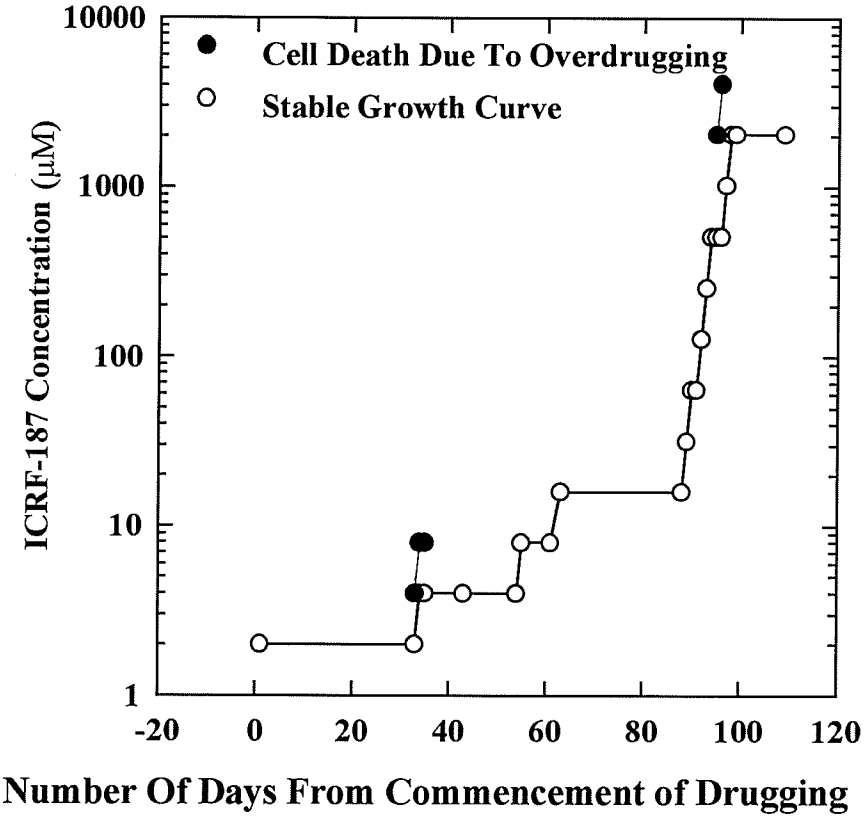


Figure 5.2.1.3.1 **The Overall Drugging Schedule Maintained In The Selection Of ICRF-187-Resistant CHO-AA8 Clones**

5.2.2 Isolation Of Single Cell Colonies Of ICRF-187-Resistant Cells

To begin selection of a single cell colony, one vial of frozen resistant cells was thawed and delivered to 10 mL of cell culture medium and allowed to grow. After several passages from stock frozen in liquid nitrogen, the cells were trypsinized (0.25% + 1 mM EDTA) and after counting, a 100 µL aliquot was serially diluted 1:10 until there were only 100 cells per mL. This 1 mL volume was now further delivered into 9 mL volume of previously used medium, which was filter sterilized to remove debris and extraneous cell material. The reason for growing these cells in previously used medium was to enhance the growth conditions of cells. The population cells that has grown in this medium has produced growth factors, which are of benefit to growing cells. It is not difficult for a large population to produce an adequate amount of these factors; however this may be difficult for populations which begin with a single cell. Thus, previously used medium would supply factors previously produced by a large number of cells. Prior to using this medium, it was filter-sterilized with a 0.2 µm syringe filter to remove any wildtype or ICRF-187-sensitive cells since this would contaminate the resistant population with wildtype cells. This medium was also supplemented with additional iron enriched and supplemented calf serum to give total calf serum concentration of 20% (v/v). Thus, the final volume was 10 mL and the cell count was 10 cells per mL. The cell suspension was mixed thoroughly and drawn up into an Eppendorff repeater pipette. The barrel used was one able to give a single delivery volume of 100 µL. 100 µL volumes were delivered into all 96 wells of a 96-well microtitre plate, giving a theoretical cell number of one cell

per well. The plates were placed into the incubator and were allowed to grow in darkness at 37°C. and 5% CO_2 and 95% air (v/v). The cells were allowed to grow undisturbed for four days and then each well of the plate was examined under an inverted phase contrast microscope to determine and mark out the wells which contained only a single colony which has arisen from a single cell. After several days, many of the 96 single cells or their colonies died, while six continued to grow well.

When these six wells of the microtitre plate were almost confluent, the cells were trypsinized with 100 μL of 0.25% trypsin and quenched with 1.9 mL of αMEM + 10% serum (v/v). The number of cells was small, and to avoid unnecessary cell death and cell loss due to stresses of centrifugation, the cells were simply delivered to six wells of a 24 well plate and were allowed to grow under the previously described conditions and in fresh medium and serum. Though the lack of washing out the excess trypsin made it slightly more difficult for the cells to anchor well, they did anchor and they were washed the following day with Dulbecco's Phosphate Buffered Saline (DPBS) and fresh medium plus serum was introduced.

5.2.3 Selection For Robust ICRF-187-Resistant Strains of CHO-AA8

The six surviving clones required more specific identification thus, DZR (denoting Dexrazoxane-Resistant) was used as the assigned abbreviation. When six clones, DZR1-DZR6, had grown to confluence in the 24-well plate, they were passaged and each group of clones was delivered to a 25 cm^2 plate to continue growing. At this stage, the cells

were subjected to daily drugging where the ICRF-187 concentration was 2 mM. Of these six clones, two had survived the drug doses and the sparse growth conditions of a plate with a small number of cells. These were the DZR2, and DZR3 clones.

For the purposes of further research it was impractical to continue work with two clones. Thus, measurements were made of the growth rates to determine which clone grew rapidly and anchored well. These simple parameters were selected as reasonable endpoints for a healthy cell.

5.2.4 Cryogenic Storage of ICRF-187 Resistant DZR2 and DZR3 Clones

The procedures described for DZR2 and DZR3 were performed simultaneously, however, for the sake of simplicity the description will be written as if it concerned only one of the two clones.

The procedure for cryogenic freezing was as described in 2.3.8. Once a plate of ICRF-187-Resistant cells was grown to a level where they were able to grow comfortably in 2.0 mM ICRF-187 the cells were propagated and 10 cryovials containing 1.0 mL (5.0×10^6 cells mL^{-1}) were preserved in liquid nitrogen. As mentioned above, the cells were being grown during the freezing period to determine if the cells would survive the freezing. Though this was tested before with the stock resistant line, it was unknown whether the cells derived from a single clone were derived from a clone able to withstand the freezing.

5.3 DETERMINATION OF VIABILITY OF THE DZR2 AND DZR3 CLONES AFTER CRYOGENIC STORAGE

5.3.1 INTRODUCTION

The initial step in the process of characterization of the DZR3 clone is the determination of the growth rate and viability of the clone after it had been subjected to cryogenic storage. This is not the same as determining the growth rate as in section 5.5. The growth rate proceeds at a rate characteristic for the cell line, but does not consider cell death or lack of the ability to grow of the weaker portion of its population. Measurement of viability accounts for only the events occurring within the first 24 hours after the cells are seeded from frozen stocks. It defines how many cells survived the first 24 hours after thawing and seeding. It does not consider the events of the survivors beyond 24 hours. This one of the steps in characterizing the CHO-AA8-derived clones which have been grown in 2.0 mM ICRF-187. It was not known prior to this experiment whether or not the extreme drug concentrations have altered the clone's ability to be thawed from liquid nitrogen and grow with the normal 90% or greater viability which is characteristic of the CHO-AA8 cell line, or whether their viability would be severely impaired.

5.3.2 METHODS AND MATERIALS

In order to determine the viability of these cells after cryogenic freezing, the cells were removed from the cryogenic tank and thawed rapidly in a 37°C water bath. The cells

were pipetted out of the cryovial and delivered into 9 mL of cell culture medium.These cells were swirled gently for several seconds to distribute them properly in the medium and then they were counted. Since the delivery of 5 million cells in 1 mL of freezing solution into 9 mL of medium would not dilute it sufficiently for seeding into a T-flasks, dilutions were made to deliver these cells to flasks for determination of viability.

Three flasks were seeded, each at a different concentration. Since it was not known how many, if any, had survived the freezing three concentrations were seeded, using the 10 mL stock. The first was seeded with 1 mL cell suspension + 9 mL cell culture medium. This was considered a low concentration seeding to be counted in the even that viability was high. A 2 mL cell suspension + plus 8 mL medium flask was seeded to measure lower viability, while providing for a countable and statistically acceptable number of cells for counting. Finally, a 5 mL suspension was seeded with 5 mL of medium in the event that viability was extremely poor.

Immediately after the seeding of each flask, 100 μL samples were removed from each flask and counted four times. After 24 hours had passed 100 μL samples of the medium were taken from each of the flasks and the number of free-floating cells were counted. The free-floating cells from each flask were counted four times and averaged. After this, medium was pipetted out of each flask and each was washed with 10 mL DPBS and the solution was pipetted out. 1 mL of 0.25% trypsin was added to each flask and the cells were dislodged. The trypsin in each flask was quenched with 9 mL of cell culture medium. 100 μL samples were removed from each of these flasks, diluted with

equal volumes of 0.6% trypan blue and the cells counted four times. Note that the cells were not centrifuged and resuspended after trypsinizing, since this step results in the loss of a large number of cells. It is also important to note that the trypsinizing, quenching, and counting steps were performed one flask at a time to minimize the possibility of trypsin damage to the cells.

5.3.3 RESULTS

The cell counts for the initial seeded flasks were 34,700, 69,400, and 173,700 for the 1 mL, 2 mL, and 5 mL seed volumes. The cell counts of the supernatants and dislodged anchored cells from each of the above mentioned flasks is tabulated below in Table 5.4.3.1. The cells were counted on a haemocytometer on five frames. The counts were recorded from both the supernatant and the dislodged anchored cells. These counts were averaged within their own category and then each was multiplied by 4,000 to account for the chamber volume and the dilution factor in the trypan blue. (See 2.1.4) After the number of cells mL^{-1} was determined, the average number of cells per millilitre in the supernatant of the flask was added to that of the anchored cells. The SEM values for both the supernatant and the dislodged anchored cells were also added together to give the total SEM. Together these values comprised the total number of surviving cells and the relative error in this determination.

Table 5.3.3.1

Cell Counts For Flasks 1, 2, and 3 After 24 Hours Anchoring

Flask Number	**Flask 1**		**Flask 2**		**Flask 3**	
Cell Source	**S**	**A**	**S**	**A**	**S**	**A**
Cells Counted*	8,000	26,000	8,800	53,000	28,000	139,000
SEM	11,200	10,000	8,400	4,000	5,600	4,000
TOTAL CELLS ± **SEM** (supernatant and anchored)	34,000 ± 21,200		61,800 ± 12,400		167,000 ± 8,400	

* Cells were counted on five frames and multiplied by 4,000 to account for dilution and chamber volume.
A = Anchored **S** = Supernatant **SEM** = Standard Error of the Mean

The initial intention to seed cells in three different concentrations in order that at least one flask carried a suitable number of countable cells to give the data statistical validity proved valuable. Though, flasks 1 and 2 gave similar values to flask 3, the errors were higher. This was likely due to the low counts observed in the cell counting stage. In flask 1, the standard deviation was roughly 50% of the cell count. In flask 2 the error value was only 20%. In flask 3, where the cell counts were higher and more correctly representative of the actual value with correspondingly more representative errors, the error was only 4.8 % of the cell count.

Because of the larger number of cells that could be counted, the values determined for Flask 3 were undoubtedly more statistically credible that those of Flasks 1 and 2. The percent survivors as a function of the number of cells seeded is listed in Table 5.3.2

Table 5.3.2

Percent Of Surviving And Viable Cells As A Function Of Cells Seeded

Flask Number	Number of Cells Seeded (mL^{-1})	SEM	Number of Cells Recovered (mL^{-1})	SEM	Percent Viability
Flask 1	34,700	3,500	34,000	21,200	97.9 ± 56.6
Flask 2	69,400	7,000	61,800	12,400	89.0 ± 17.9
Flask 3	173,000	18,000	167,200	8,400	96.3 ± 4.8

Though these value show reasonable agreement, those of flask 3 carry the lowest margin of error and the percent viability of the cells is therefore most reasonable assumed to be 96.3 ± 4.8 %.

DISCUSSION:

The viability of the DZR3 clone after cryogenic storage was shown to be as high as 96.3 ± 4.8 %. Though, the values obtained from all of the flasks are comparable (Flask 1: 97.9 ± 56.6 %; Flask 2: 89.0 ± 17.9; Flask 3: 96.3 ± 4.8 %) the latter, seeded with the largest number of cells gave the most reliable information. This viability experiment

showed that the drugging and selection of an ICRF-187 resistant clone had no effect on the ability of the DZR3 line to survive cryogenic storage.

5.4 MEASUREMENT OF THE GROWTH RATES AND DOUBLING TIMES OF THE DZR2 AND DZR3 CLONES

5.4.1 INTRODUCTION

One of the characteristics that must be determined when a new cell line has been selected is the growth rate of the cell line. This information is not only valuable for the sake of characterizing the cell line, but more importantly it is a tool useful in growing cells for experiments. It allows the investigator to predict the number of cells that will grow from the time of seeding, considering both lag and doubling times. Alternatively, it allows the investigator to compute, with the same knowledge of lag time and doubling time, the number of cells required in seeding to generate the number of cells required for an experiment a number of days in advance.

The comparison of growth rates was made between both DZR2 and DZR3 resistant clones in order to compare the stability or even the fragility of the two clones. This information was used to select the primary resistant line which was to be used for research into drug ICRF-series and BLPD-series cytotoxicity experiments, cross-resistance experiments, as well as experiments to test inhibition of topoisomerase II and P-glycoprotein functions in the resistant and wildtype cell lines.

5.4.2 METHODS AND MATERIALS

Both DZR2 and DZR3 were grown drug-free for 2 passages in two 75 cm^2 flasks. The following protocol was used for both cell lines simultaneously. The cells were trypsinized, quenched with cell culture medium + 10% calf serum (v/v), and centrifuged. The supernatant was discarded and the pellet was resuspended in 10 mL of cell culture medium + 10% calf serum (v/v). The cell suspensions were each counted six times. After counting, each cell line was seeded into six 25 cm^2 T-flasks at 40,000 ± 4,000 cells mL^{-1} in a total of 10.0 mL. Thus, at zero time the cell count in each of the six flasks per cell type was 40,000 ± 4,000 cells mL^{-1}. The cells were allowed to grow in their flasks and at a given time interval, one flask from each cell line was trypsinized, quenched, centrifuged, resuspended and counted to give the number of cells per mL in a total of 10.0 mL of medium. This was done at 12, 24, 48, 72, 96, and 120 hours after seeding. The cells in each of the counting procedures were counted ten times each per flask per time interval. Counts were taken for the free-floating cells in the supernatant as well as the anchored cells. These values and their respective standard deviations were summed to give a total number of (cells ± error) mL^{-1} for both cell lines, for each time interval. This information is tabulated in Table 5.4.3.1. Figure 5.4.3.1 illustrates the growth curves of DZR2 and DZR3 over the course of 120 hours.

5.4.3 RESULTS

The growth of both DZR2 and DZR3 is summarized in Table 5.4.3.1. From this data, Figure 5.4.3.1 was constructed to show the growth characteristics of both cell lines.

Table 5.4.3.1

The Sum Of Cell Counts Of Free-floating And Anchored DZR2 And DZR3 Cells Over 12, 24, 48, 72, 96, And 120 Hours Of Growth.

Time (Hours)	DZR2 Cell Count (Cells mL^{-1} x 10^3)	SEM x 10^3	DZR3 Cell Count (Cells mL^{-1} x 10^3)	SEM x 10^3
0	40.0	4.0	40.0	4.0
12	48.0	15.5	65.6	14.7
24	50.0	21.8	84.6	22.3
48	154.4	30.8	250.8	45.6
72	505.2	58.9	668.0	97.8
96	914.0	156.1	1230.0	96.6
120	1850.0	251.3	1460.0	150.2

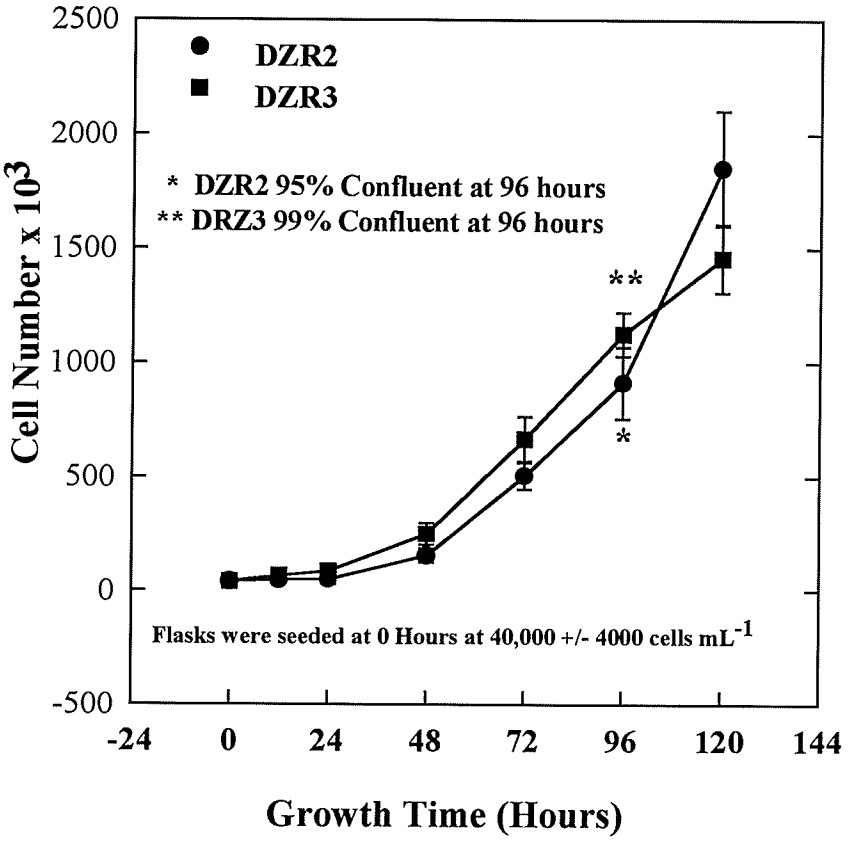


Figure 5.3.1 Growth Curves Of DZR2 And DZR3 Over The Course Of 120 Hours

5.4.4 DISCUSSION

Figure 5.4.3.1 illustrates the growth curves of both resistant cells lines. As can be seen by the two growth profiles, there was little difference in the actual rates of growth once the cells began growing in the exponential phase. The greatest difference in the growth appeared in the first 24 hours of the growth curve. Though, both cell lines were seeded at 40,000 cells mL^{-1}, DZR2 had grown to 48,000 cells mL^{-1} in the first 12 hours and to only 50,000 cells mL^{-1} in the first 24 hours, while DZR3 grew more rapidly from 40,000 mL^{-1} to 65,500 mL^{-1} and finally to 84,600 cells mL^{-1} in 24 hours. This represents an increase of 25% in the number of DZR2 cells grown in the first 24 hour increment, while in increase of 112% was seen in the DZR3 cells in the same time. At the 48 hour time point, the DZR2 cells had increase by 186% of the initial count and the DZR3 cells had increased to 527% of the initial count. The growth rate progressed equally well for the following 48 hours. Though the numbers of cells differed, the growth rate was the same, as is shown by the slope of both curves. After the 96 hour point, the number of cells counted in the DZR2 flask surpassed those in the DZR3 flask. Inspection under the microscope shows that at the 96 hour time point, the DZR2 cells were approximately 95% confluent, with a small amount of room still remaining for cell growth. The DZR3 cells however, were at the point of 99% confluence, with no room left for growth. At this stage, cell growth at the exponential rate stops due to contact inhibition, and cell death will ensue due to build up of toxins, and depletion of nutrients. The DZR3 began dying out while DZR2 cells were still growing well. This resulted in a decrease in the number

of the rapidly growing DZR3 cells at the 120 hour mark.

Though there was an increase of approximately 112% in the DZR3 cell number within the first 24 hours, there was also a 17% error in this number. For the most part there was a substantial increase in the number of cells, but when compared with CHO-AA8 wildtype cells, this increase is a relatively small one. Experiments such as these were conducted to verify growth characteristics of the CHO-AA8 cells (data not shown). Growth within the first 24 hours increased from 40,000 to 116,400 cells mL^{-1}. This increase in cell number represents an increase of 191% over the original number of cells seeded in the flask. There is a difference between the abilities of the wildtype and resistant cells to begin rapid growth. These data suggest a lag time of approximately 12 hours for the AA8 cells and 18-24 hours for the DZR3 cells.

The selection of a "primary" cell line to be used for drug experiments was based predominantly on simple criteria such as overall growth rate, and time required for transition from lag phase growth to exponential growth. From the data shown in Figure 5.4.3.1, there is little evidence of a substantial difference between the cell lines. As discussed above, the only real difference is in the inial 24 hours where there is a slightly quicker initiation of growth in the DZR3 flask than in the DZR2. This may be interpreted as being a slightly healthier or heartier cell line. For this reason DZR3 was selected as the cell line for further drug cross resistance experiments with bisdioxopiperazines, certain anthracyclines and an array of antitumor drugs.

The doubling time of these cells was computed using the exponential equation:

$$N = N_0 e^{\kappa\tau}$$

The numbers used to compute the growth rate were N_0 the number of cells per mL at the time of seeding, (*i.e.* 40,000) and those at the end of the experiment, N. The number of cells at 120 hours of growth was 9.14×10^5 cells mL^{-1}. These numbers were selected since each increment between time points had different growth rates. The selection of the correct overall rate of growth was not possible, thus the overall average rate was used, including the lag time. The number of cells counted at the 120 hour time point was not used for the calculation of the growth rate constant or the doubling time since the cells had already passed confluence at this point and were in decline. This was not representative of the true growth potential of the cells, and so the value was not included. Only time periods which included exponential growth were used for the calculation. It was first necessary to determine the value of κ, the rate constant, before the doubling time τ, is found. Substitution of N_0 with 40,000 and N with 9.14×10^5 and substitution of τ with 96 hours gives $9.14 \times 10^5 = 40{,}000\ e^{\kappa 96}$. Solving for κ gives an overall growth rate constant of 0.0326. Substitution of this κ value into an equation with 2 and 1 substituted into N_0 and N respectively, allows for the solution of τ:

$2 = e^{0.0326\tau}$ and solving for τ gives an overall doubling time of 21.3 hours. A doubling time of 21.3. hours is roughly 1.8 times the doubling time of the wildtype CHO-AA8 cells of 12 hours. This indicates that there is a change in the metabolic properties and functions of the resistant clone as compared with the parental line.

5.5 RETENTION OF ICRF-187 RESISTANCE BY DZR3

5.5.1 INTRODUCTION

Once a cell line has been selected to be resistant to a particular agent or group of agents, it is valuable to know the duration of resistance of the cells to the agent. Experimental evidence regarding differences between the parental cell and the resistant derivative is only valid when the cell is functionally resistant. Most often, investigators maintain the resistant phenotype of their resistant cell line by challenging the line to the selection drug on a monthly basis. A portion of the resistant phenotype is passaged routinely in the absence of drug and tested on a monthly basis along against the drug-challenged phenotype to compare the stability of the resistance in the cell line that has not been routinely challenged (Yang and Trujillo, 1990).

Once the stability of the DZR3 cell line had been established with respect to cryogenic freezing and the questions of growth rate and viability were addressed, the stability of ICRF-187 resistance was tested.

5.5.2 METHODS AND MATERIALS

DZR3 cells that were passaged two to three times weekly were challenged once a month with ICRF-187 to ensure resistance of the cells to the drug. The cells were propagated in 25 cm^2 T-flasks containing 10 mL of cell culture medium (αMEM + 10% (v/v) iron enriched and supplemented calf serum). The cells were passaged by washing with Dulbecco's PBS, trypsinizing and centrifuging the cells into a pellet at 2,000 x *g*.

The cells were resuspended in 10 mL of fresh medium and 1.0 mL of this suspension was delivered into a fresh flask containing 8.0 mL of fresh medium. A 10 mM solution of ICRF-187 was prepared in αMEM + 10% (v/v) calf serum, filtered and 1.0 mL of this drug solution was delivered to the 9.0 mL already in the flask and swirled to mix. The cells were allowed to grow to near-confluence. When the cells were not being drugged, they were simply passaged by splitting them to 1/10 in medium two to three times a week.

In testing the stability of the resistant phenotype, a portion of these cells was plated in a second flask during a routine passaging. These cells were also passaged routinely, but no drug was ever added to the medium. After one month had passed, the resistance to ICRF-187 was measured.

5.5.2.1 Seeding Of The Experiment

The cell culture medium was pipetted out, the flask was washed with Dulbecco's PBS and then trypsinized. The cells were centrifuged after delivery into a 15 mL sterile centrifuge tube. The supernatant was discarded and the cells were resuspended in 10 mL of cell culture medium and counted. The cells were counted six times and the counts were averaged. The appropriate dilution was made with cell culture medium to give a cell count of 20,000 cells mL^{-1}. Cells were delivered to two 96-well microtitre plates as described in Chapter 3. The cells were delivered in 100 μL aliquots to each well to give 2,000 cells per well. The same seeding was performed with the drug-challenged ICRF-

187-resistant cells. Both sets of plates were allowed to incubate for 24 hours in darkness, at 37°C, in an atmosphere of 5% CO_2 and 95% air (v/v). Cells were delivered to all, but six wells of each plate. These were to serve as DMSO blanks for the end-point detection method described below. All cell seeding was done using an Eppendorff repeater pipette fitted with an autoclaved 5 mL barrel set to deliver 100 µL volumes.

5.5.2.2 Preparation And Delivery Of Drugs

After 24 hours, ICRF-187 solution was prepared for dilution and drugging of four plates of cells. The range of drug concentrations was from 0.01-20,000 µM. This was to ensure a broad range of drug concentrations. Each concentration was tested in six wells for both sets of cells to ensure statistical validity. The amount of drug required for this experiment was roughly 30 mg, prepared in a stock solution of approximately 6 mL at a concentration of 20 mM. The drug was filtered using a 0.2 µm syringe filter, then serially diluted 1:10 with αMEM into 4 mL volumes ranging from 0.2-20,000 µM. The drugs were delivered as illustrated in volumes no less than 10 µL and no greater than 100 µL. The volume of those wells not containing a total of 200 µL was made up to 200 µL with cell culture medium. The single exception to these addition trends was the wells that were to contain 20 mM drug. In this case, the volume of medium remaining in the well from the seeding of the cells at the beginning of the experiment was aspirated off and 200 µL of 20 mM ICRF-187 in cell culture medium was delivered to each of the wells. All drug and medium delivery were made by using an 8 x 200 Gilson multichannel pipettor and sterile microtips. Care was taken not to touch the bottom of the wells with the pipette tips

during the drug delivery, since this would likely kill the cells touched and lead to false results. The cells were allowed to incubate with drug under the above-mentioned conditions for 48 hours.

5.5.2.3 End-point Detection

After the 48 hour incubation period, the effects of the drug were measured using the MTT end-point detection method. 20 μL of the 2.5 mg mL^{-1} MTT solution was added to each of the wells containing cell. The MTT was allowed to incubate for 4 hours under the conditions described above. After 4 hours, the cell culture medium was carefully aspirated out of each well. Aspiration was performed using an 18 gauge needle fixed to a water aspirator. The bevelled edge of the needle was lowered along the wall of the well and the medium was aspirated out with only the very tip of the needle touching the bottom of the well. This ensured complete removal of all the medium, which was important in the solubilizing of the formazan crystals with DMSO, while leaving the cells and their crystals undisturbed. Care was taken not to disturb the cells anchored to the well bottoms which contained the formazan dye.

DMSO was added to each of the wells (including those reserved as blanks) of all of the plates. After a suitable time when all of the crystal appeared to have dissolved (usually three hours), the plates were read at a wavelength of $\lambda = 490$ minus $\lambda = 650$ nm.

5.5.3 RESULTS

After the computation of the means and standard errors of the means on the plate reader, the results were plotted using the Sigmaplot 5.0 program. The IC_{50} values were computed using the equation,

$$Abs_{490\ obs} = (a-d)/(1+(x/c)^b)+d$$

where $a = Abs_{490max}$, $d = Abs_{490min}$, c = the IC_{50} value computed in the fit, b = the exponential factor, which give the rate of increase of cytotoxicity as a function of the increase in drug dose, and x values are experimental drug doses used in the experiment. These are selected from the column containing the drug concentration data in the Sigma plot program.

The calculation of the IC_{50} value for the cells grown drug free for 30 days was shown to be 2,400 ± 200 μM and after 65 days it was shown to be 1,800 ± 200 μM. The value computed after 2 passages of drug-free growth was 2,200 ± 500 μM. The represents an 8.3 % loss in the resistance of the cell to ICRF-187 after 30 days and 25% after 65 days. The experimental error in the 0 and 30 day values are large enough to shown that these values may or may not be considered as being the same. The differences in the 0 and 65 day values appear more significant as the difference indicates a 25% reduction in drug resistance with errors small enough to discount an overlapping of values.

5.5.4 DISCUSSION

The level of resistance has been shown to have decreased marginally over the course of 30 days however, the difference is not considered statistically significant. The difference observed between the 0 and 65 day experiments appear to be more significant as a 25% reduction in drug resistance is seen. Though, the acquisition of the resistant phenotype and the loss of the resistance differs for different cells and for the different drugs to which they are made resistant, the loss of resistance over a short time is in itself not unusual. In the case of a multidrug resistant variant (H69AR) of the human small cell lung cancer cell line NCI-H69, 32 fold resistance to doxorubicin and numerous anthracycline analogues was acquired after a total of 14 months of growth in doxorubicin. In determining the stability of the cells during drug-free growth, the resistance of the cells to doxorubicin had dropped to about 60% of the peak resistance within 35 days. Over the course of comparisons between the drugged population and the drug-free populations of cells, the experiment revealed that, though the first 35 days showed a 40% decrease in stability, there was no decrease below this value over the remaining duration of the experiment, which totalled 181 days. This resistance was found to have decreased for all of the anthracycline analogues tested (Mirski *et al.*, 1987). Loss of drug resistance was also observed by Twentyman *et al.*, (1986), who observed initial loss of resistance in the first three weeks of drug-free growth, but no more loss of resistance for up to 9 weeks.

For the ICRF-187-resistant cells, the loss of drug resistance is not as high as what has been found by other investigators. As was mentioned above, this may be due to high

errors in the inhibitory concentration values, but it may also be a feature of bisdioxopiperazine resistance that has not been reported by other investigators, whose work involves anthracyclines and not bisdioxopiperazines.

5.6 DESCRIPTION OF DZR3 CELL MORPHOLOGY

In the initial stages of selection of the ICRF-187 resistant cell line, the doses of the drug administered on a daily basis were 2.0 μM. Though this was close to the IC_{50} level measured for the drug with the wildtype CHO-AA8 cells, it was in the overall scope of the project, a low dose. In the initial stages of the process, often growth was slow and cell death was higher than under normal growth conditions. Trypsinizing of cells resulted in the harvesting of a small number of cells, often lower than what was optimal for seeding into a flask for growth with fresh drug. CHO-AA8 cells seeded at 30% would easily reach confluence within 48 hours, whereas the rate of growth of cells exposed to daily doses of ICRF-187 was clearly slower. Cells seeded at 30% confluence were found to have reached approximately 40% confluence over the course of 72 hours. Growth was also problematic in that the cells required trypsinizing even though they were not near confluence overall. While most of the plate showed surfaces which were approximately 40% confluent, a number of colonies would arise that were able to withstand the drug levels and these would grow fairly normally, creating clusters of overgrown cells. These cells needed to be disaggregated and disbursed for better growth. As the drugging continued, the proportion of resistant cells in a given population increased. Within the

space of three days of continuous drugging, obvious changes were apparent in the growth patterns of the cells. Where it took 72 hours for the number of cells to increase from 30-40% confluence, after three more days of drugging, the trypsinizing and reseeding of cells at 40% on one day resulted in the growth to 70% within 24 hours.

These changes in the rate of cell growth were only the first of several changes observed in the cells acquiring drug resistance. The specific reference to acquisition of resistance is made since these cells were not simply surviving or not surviving. Rather they were undergoing what appeared, under the microscope, to be physical or morphological changes. It was likely that the peripheral changes in the cell were indicative of a significant internal change in the biochemistry of the cell. Photo 5.6.1 shows CHO-AA8 cells growing in the absence of ICRF-187.

While still growing at the 2.0 μM doses of ICRF-187, the cells began to show signs of physical change. They often carried blebs or extrusions, they were often two to three times larger than wildtype cells or most of the resistant cells around them. Many that were surrounded by a halo-like shield, which was not likely to be a microscopic artefact. These cells appear clearly under the microscope when they are in a flask and growing in their medium, but clarity is often lost in the process of staining and photographing of the cells. In a group of nearly confluent cells one large cell was alone in the middle of a large aggregate of cells. When observed under higher magnification, this cell had a large cell membrane extending from the nucleus in a radial distance two to three times greater than that seen in the cells around it. (Photo 5.6.2) This would occur

in all periods of cell stress, where doses of drug were doubled after a period of comfortable growth in a certain level of drug and especially if the cells were grown for a period of days without drug. One problem that is clarified in the staining process is the nature of the halo-like cover surrounding the oversized cells. The staining process surrounds the cells and is able to colour the cytoplasmic material and differentiate it from the nuclear material. In the oversized cells, the stain is able to penetrate the cell and the nucleus, but the halo-like structure does not take up the stain. The cells appear separated from each other, but surrounded by a yellowish wax or paste, which under growth conditions in the T-flask, appears as though the cell is surrounded by a halo. What appears to be a large multinucleated cell in the cell culture medium is actually several cells surrounded by a wax-like substance. The actual nature, physical or chemical, is not known. The reintroduction of drug, at higher concentrations resulted in a small number of cells that appeared larger than usual or often very slender, but also very long and resembling a dendrite and rather than an ovary cell. The most striking feature observed was a multinucleated cell. Though this was not an uncommon occurrence, especially at times when drug concentrations were doubled, these cells were difficult to photograph. It was often difficult, especially after staining, to differentiate between large cells and large multinucleated cells. Many cells appeared binucleated, but this could have been due to cell death caused by the staining during a point immediately prior to cytokinesis, and not necessarily a cell which is being affected by the drug. One photograph was made at the point where cells subjected to drug were for the most part, used to the extreme doses

of the drug. This made capturing an image of a multinucleated cell difficult since the cells were no longer as likely to be affected by the drug. Photo 5.6.3 shows a DZR3 cell grown for 12 hours in 3 mM ICRF-187.Notice the oversized cells. Though it may be possible that a number of these are some type of microscopic artefact, it is not likely since there are no such artefacts observed in other photographs or in cell culture flasks growing with undrugged cell culture medium. Photo 5.6.4 shows what appears to be a multinucleated cell. The cell is not only larger than the cells around it and larger than wildtype cells, but it appears to contain approximately ten nuclei.

The effects manifested are seen only in the presence of ICRF-187, and not when the cells are growing under drug-free conditions. This is true for both the wildtype and resistant cells that have been drugged after growing drug-free for several passages. The possible role of ICRF-187 in the arresting of mitosis has been discussed in Chapter 3.

The following photographs were made at 200 x magnification using Kodak 200 ISO Kodacolor film. Cells were stained with Camco Quickstain II Buffered Differential Wright-Geimsa stain.

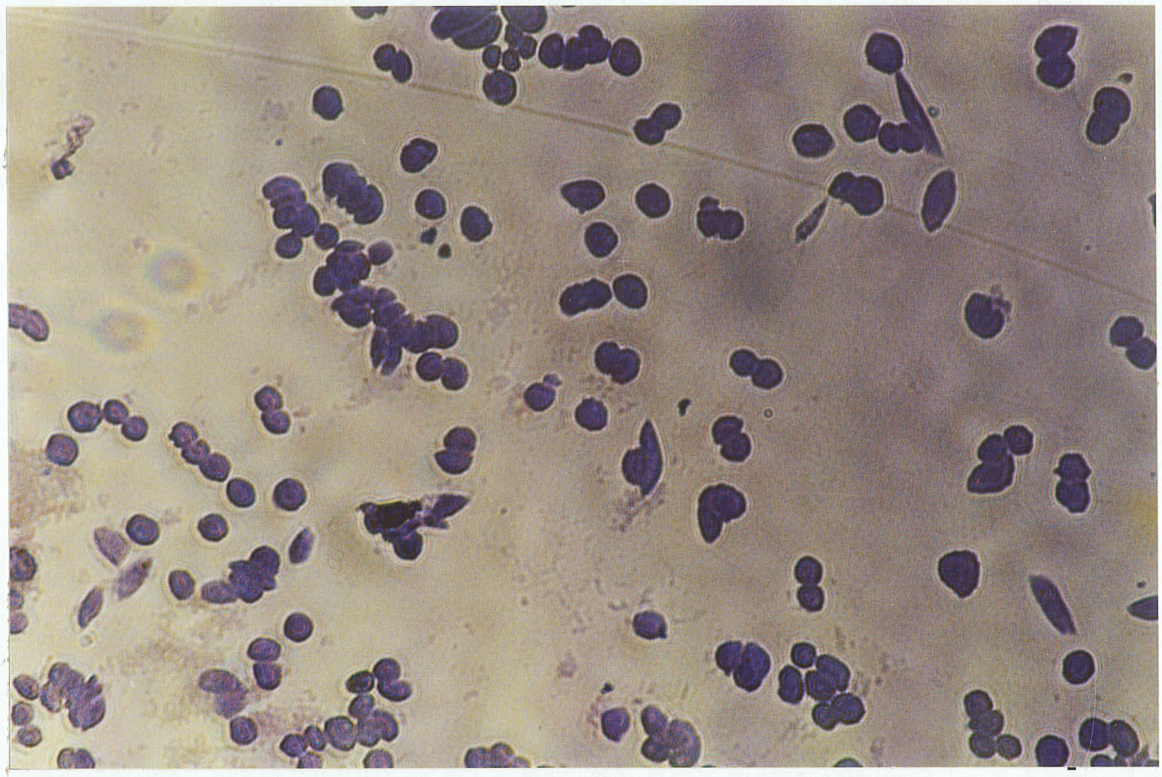


Photo 5.6.1 CHO-AA8/No Drug

Photo 5.6.1 shows the CHO-AA8 wildtype cell in the absence of ICRF-187. The rounded cells were likely completing cytokinesis and have not yet anchored. The more angular cells are well anchored cells between reproductive cycles.

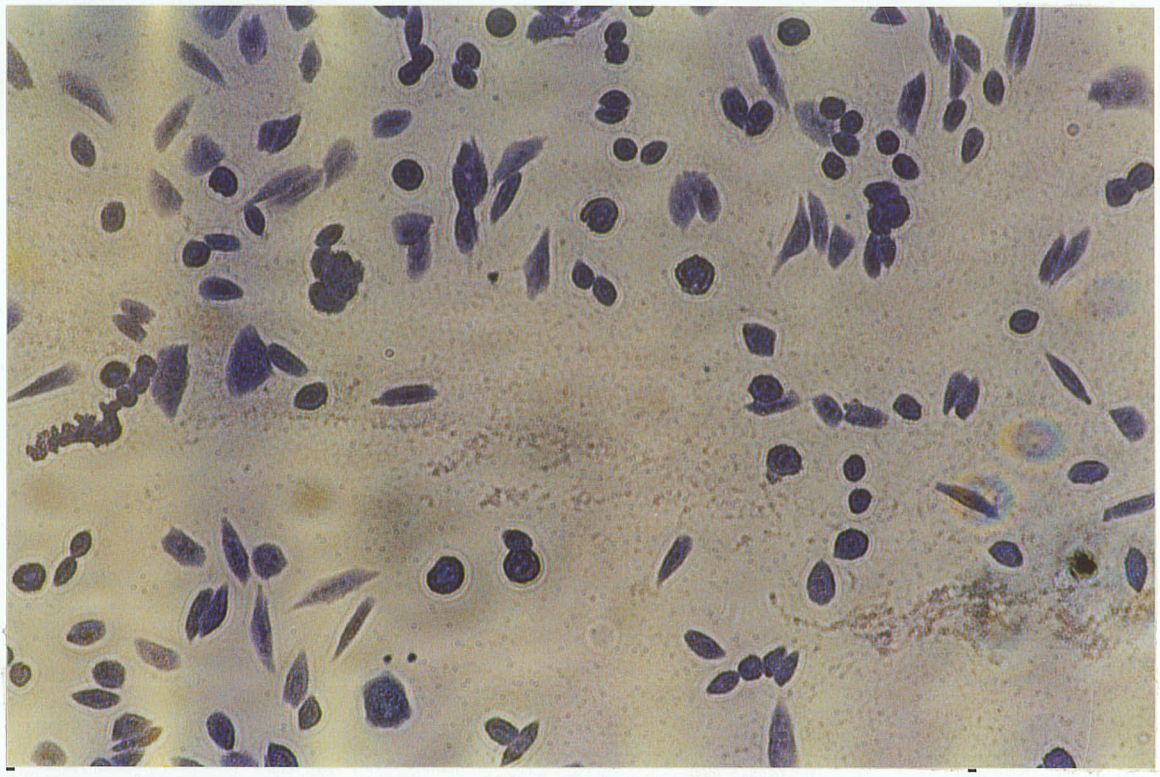


Photo 5.6.2 DZR3/No Drug

Photo 5.6.2 shows the ICRF-187-Resistant clone, DZR3 in the absence of ICRF-187. In the absence of ICRF-187, these cells appear much the same as the wildtype though, several appear slightly larger.

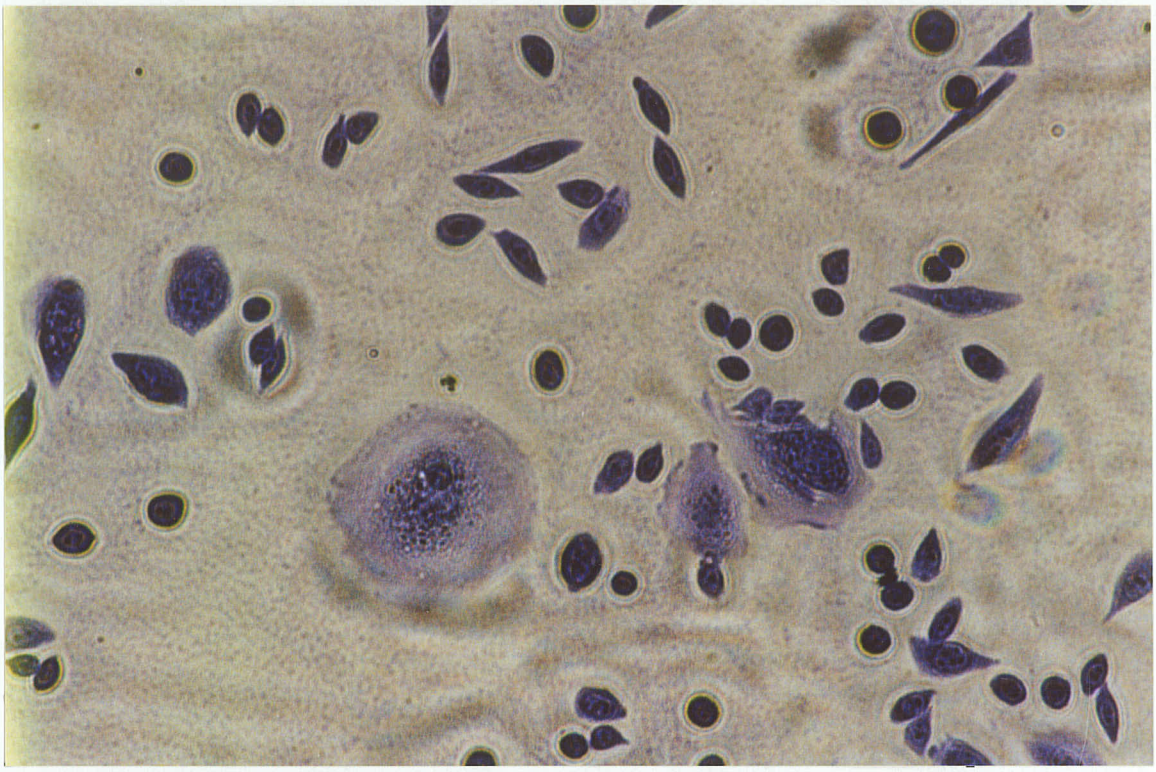


Photo 5.6.3 Oversized DZR3 Cells Growing In 3.0 mM ICRF-187

Photo 5.6.3 shows the normal DZR3-type structure with some rounded cells, many of the angular-shaped anchored cells growing in the presence of oversized cells. Though, three of the oversized cells are 2-4 times as large as the normal DZR3 cells, one is at least 10 times the size of a normal resistant cell. These cells were grown for several passages without any drug and were exposed to ICRF-187 for a period of 12 hours prior to being photographed.

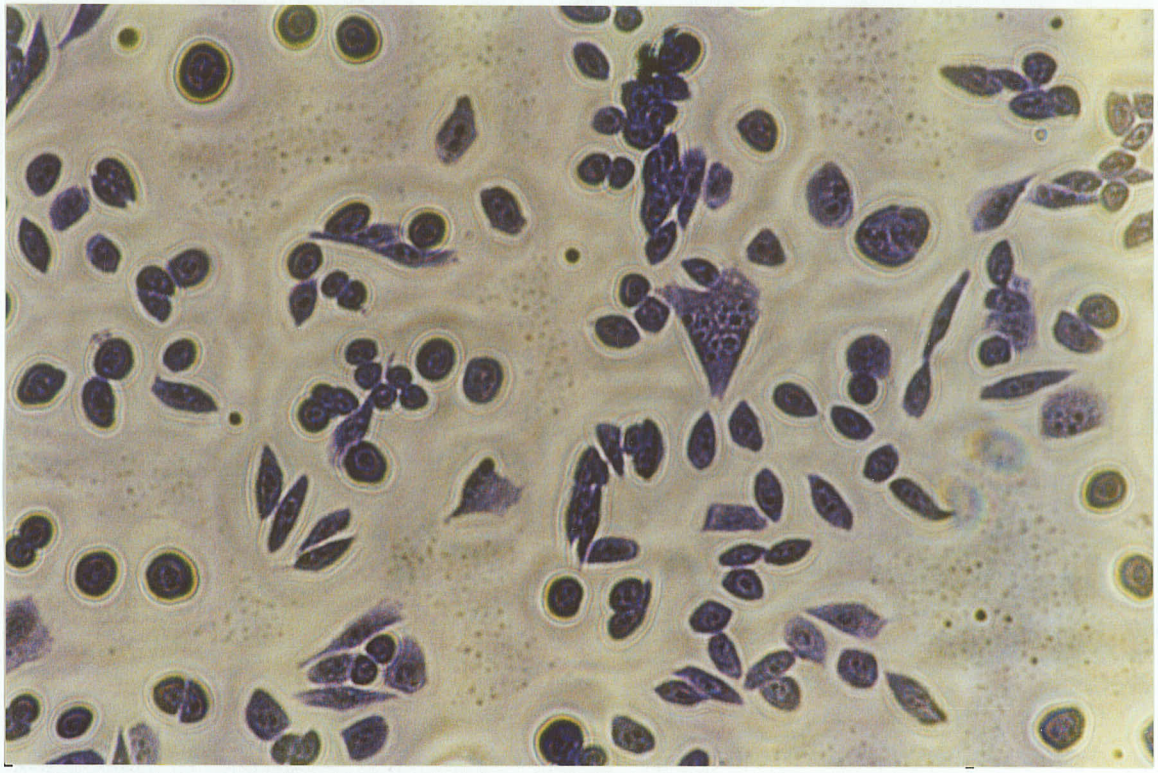


Photo 5.6.4 Multinucleated DZR3 Growing In 3.0 mM ICRF-187

Photo 5.6.4 shows a single multinucleated cell growing in the presence of a host of normal DZR3 cells. This cell appears to be carrying as many as 10 nuclei. Unlike the large cells discussed above, which appeared multinucleated only in the flask, but not after staining, this photo clearly shows the large number of nuclei in a single cell. Note that there is no surrounding halo or shell. These cells were grown for several passages without any drug and were exposed to ICRF-187 for a period of 12 hours prior to being photographed.

Among the physical changes observed were differences in the adhesiveness of the resistant cells The resistant line appear to be anchored more solidly to the T-flasks than do the sensitive counterparts. When sensitive and resistant cells are being trypsinized to remove them from the T-flask, trypsinizing the resistant cells usually requires more time and often more aggressive agitation of the flask than do the sensitive cells, even though the same amount of enzyme was used on both flasks, and both flasks are at the same degree of confluence. Multidrug resistant Chinese Hamster lung fibroblasts grown by Biedler *et al.*, (1975) manifest the same adhesiveness, as do the doxorubicin resistant H69AR cells grown by Mirski *et al.*, (1987).

5.7 MEASUREMENT OF CROSS-RESISTANCE TO CYTOTOXIC DRUGS TESTED ON CHO-AA8 CELLS AND DZR3 CELLS

5.7.1 Resistance To Bisdioxopiperazine Compounds

5.7.1.1 INTRODUCTION

The introduction of the ICRF series bisdioxopiperazines as a new class of antitumor agents which affect the topoisomerase II enzyme revealed a previously unknown mechanism of inhibition (Tanabe *et al.*, 1991). Studies have identified the inhibition of topoisomerase by antitumor agents to occur through the induction of cleavable enzyme complexes. The drugs would interfere with the breakage-reunion reaction of DNA topoisomerases. In the presence of these drugs, an aborted reaction intermediate was formed, known as a cleavable complex. These are denatured DNA products with both single-strand and double-strand breaks in the DNA in which the topoisomerase II subunit is covalently linked to the 5'-phosphoryl end of each broken DNA strand. Topoisomerase II, specifically, is essential for segregation of replicating yeast daughter cells (Ishimi *et al.*, 1992). The identification of these complexes as the source of DNA lesions resulting from the effects of the topoisomerase-targeting drugs readily explains the overall cellular effects observed when these drugs are administered (D'Arpa and Liu, 1989).

The ICRF series bisdioxopiperazine compounds, specifically ICRF-154, ICRF-159, and ICRF-193, were able to inhibit mammalian topoisomerase II activity. However, the mechanism did not involve intercalation of the compounds into the DNA to form a

cleavable complex. Rather, the most potent of the three, ICRF-193, was shown to be able to bind the topoisomerase directly and inhibit its function. (Tanabe *et al.*, 1991)

The DZR3 cell line was developed as an ICRF-187-Resistant derivative of the CHO-AA8 cell line. Over the course of 110 days this cell line acquired substantial drug tolerance over the initial resistance of the parent line to the drug. Where the initial IC_{50} of the parental cell line was 2.7 ± 0.2 μM, the IC_{50} of the resistant cells was in the order of 2400 ± 200 μM. This represents an increase in tolerance for ICRF-187 between the sensitive and resistant lines in the order of roughly 900 fold. Experiments were conducted to measure whether or not any resistance was acquired for the analogues of ICRF-187. Often when cells become resistant to an antitumor agent, they soon begin to show cross-resistance to many other drugs.

The drugs tested in this set of experiments included only those bisdioxopiperazines which showed a high degree of cytotoxicity with the wildtype or sensitive CHO-AA8 cells. These included ICRF-154, ICRF-159, ICRF-161, ICRF-186, and ICRF-193. These represent the most toxic of the ICRF-187 analogues tested. The purpose of this set of experiments was to compare and contrast the cytotoxicity and resistance to cytotoxicity between the sensitive and drug-resistant cells in order to determine whether there is a structural relationship among analogues which would result in any degree of cross-resistance.

5.7.1.2 METHODS AND MATERIALS

5.7.1.2.1 Seeding Of The Cells Into The Microtitre Plates

The experiments conducted to measure cytotoxicity among the bisdioxopiperazine analogues were conducted simultaneously with both the sensitive CHO-AA8 cells and the resistant derivative, DZR3. Cells were seeded into two sets of 96-well microtitre plates, two plates per set of cells. Each set of plates tested drugs on both sets of cells to the limit of 20 mM or to the limit of solubility of the drugs. The cells from the two cell lines were seeded into the wells at different concentrations. In the singe cell-line type experiments described in previous chapters, there was adequate growth in the cells and sensitivity in the endpoint detection to observe and calculate accurate IC_{50} values. The lag phase of the DZR3 clone is from 18-24 hours and its doubling time is roughly 21 hours. The sensitive cells had a lag period of only 12 hours before entering their exponential growth phase and had a doubling time of approximately 12 hours. With the seeding of DZR3 cells for experiments along with the CHO-AA8 cells, there was a significant difference in the growth rates and lag phases of the two cell lines to create problems in the interpretation of the information. For this reason experiments needed to be seeded at different concentrations to give comparable cell numbers for measurement of toxicity at the end of the experiment. The number of cells remaining alive after the experiment had to be detectable by the spectrophotometer, but at the same time the controls could not exceed the limit of detection of the spectrophotometer. In order to give an adequate number of cells that were detectable by the spectrophotometer and produce an accurate cytotoxicity

profile, the number of DZR3 cells seeded into each well was 5,000. Since the sensitive cells had a lag period of only 12 hours before entering their exponential growth phase and had a doubling time of approximately 12 hours, they were seeded at 2,000 cells per well. As in previous experiments, cells were seeded and allowed to incubate for 24 hours in darkness at 37°C, in an atmosphere of 5% CO_2 and 95% air (v/v).

5.7.1.2.2 Preparation Of Drugs

Delivery of drugs to the plates was often complicated by two problems. In order to measure the cytotoxic effects of drugs on a line of cells that was resistant, the drug concentrations needed to be much higher than they would be in the sensitive cell experiments. The fact that these experiments consumed large quantities of drug was in itself not an obstacle in the experiment, however, the fact that a number of these drugs were not very soluble in αMEM was a formidable problem. Testing the toxicity of a poorly soluble drug at high concentrations in an aqueous environment required that these drugs be dissolved in DMSO as was described in Chapter 3 and delivered into wells in 1.0 μL volumes. ICRF-193, which was poorly soluble in αMEM was dissolved in DMSO at a concentration of 10 mM and delivered to the cells in a volume of 1.0 μL. The resulting concentration in the well, which contained 200 μL of medium in total did not exceed 50 μM however, this was an adequate concentration considering the extreme toxicity of the drug.

The drugs which were more easily or more suitably soluble in aqueous solutions were prepared in cell culture medium and did not require any special preparation other

than sonication for 20-30 minutes. These included ICRF-159, ICRF-186, and ICRF-187. Each of these drugs was prepared to their respective limits of solubility. Since ICRF-154 and ICRF-161 were not easily soluble in αMEM at high concentrations, they were dissolved in DMSO. ICRF-154 dissolved readily in DMSO however, ICRF-161 would dissolve only after 10-20 minutes of sonication. These were diluted as described in Chapter 3 and were delivered in 1.0 μL volumes to the wells of the microtitre plate and made up to 200 μL by adding 99 μL of αMEM to each well.

5.7.1.2.3 Delivery Of Drugs

Where drugs were not delivered in 1.0 μL volumes in DMSO to a well containing 199 μL of cell culture medium, they were delivered in volumes not less than 10 μL and not greater than 100 μL. Volumes that were less than 100 μL were made up to 100 μL with cell culture medium to give a total drug delivery volume of 100 μL and a total well volume, including seeding volume of 200 μL. Drug preparation and delivery was made based on the correct final concentration and a final well volume of 200 μL.

5.7.1.2.4 End-point Detection.

Following drug delivery, the cells were allowed to incubate for 48 hours in the above-mentioned conditions. After incubation with drug, 20 μL of 2.5 mg mL^{-1} MTT was added to each well and allowed to incubate for an additional 4 hours. The medium was then aspirated off, the formazan crystals were dissolved in 100 μL spectral grade DMSO and the plate was read on a spectrophotometer at λ= 490 minus λ = 650 nm.

5.7.1.3 RESULTS

The results of the study comparing the cytotoxic effects of several ICRF series bisdioxopiperazines, namely ICRF-187, ICRF-154, ICRF-159, ICRF-186, ICRF-193, show the effects manifested on wild type cells as well as on the resistant strain of cells. The differences in the degree of insult will be compared in the case of each drug with regard to both cell lines and a degree of resistance for each drug will be established.

5.7.1.3.1 ICRF-187

In the comparison of the degrees of resistance manifested by the resistant cell line, the drug concentrations for the assay were increased from 0.01-50 μM to 0.01-10,000 μM, spanning a range of 6 log units. Both cell lines were assayed at the same time under the same conditions. The increased resistance in the DZR3 line was as expected, much higher than that seen in the sensitive line however, the actual profile shown in the sensitive line was not expected. One of the most interesting features of the ICRF-187 cytotoxicity profiles examined was that the CHO-AA8 cells had what appeared to be two levels of resistance or plateaus of drug tolerance and consequently, two IC_{50} values. The IC_{50} values were computed using the equation,

$$Abs_{490obs} = (a-d)/(1+(x/c)^b)+d$$

where $a = Abs_{490max}$, $d = Abs_{490min}$, c = the IC_{50} value computed in the fit, b = the exponential factor, which give the rate of increase of cytotoxicity as a function of the increase in drug dose, and x values are experimental drug doses used in the experiment. These are selected from the column containing the drug concentration data in the

Sigmaplot program. The higher of the two IC_{50} values corresponded favourably to the single IC_{50} value observed in the resistant cell line.

Figure 5.7.1.3.1A shows the cytotoxicity profiles of the CHO-AA8 cell line. These cells have an IC_{50} value of approximately 2.0 ± 0.2 μM when computed between drug concentrations of 0-700 μM. When computed in an overlapping range from 70-10,000 μM, the IC_{50} value is computed as 2400 ± 200 μM.

Figure 5.7.1.3.1B shows the MTT Assay of ICRF-187 mediated cytotoxicity after 24 hour incubation with the sensitive CHO-AA8 cells as well as with the ICRF-187-resistant DZR3 cells. For the CHO-AA8 cells, there are two distinct curves and plateaus visible in the cytotoxicity profile. The IC_{50A} value represents the value computed within the range of the first killing plateau, while IC_{50B} represents the value computed between the first plateau and the final plateau. These values are 2.3 ± 0.2 μM and 2,200 ± 200 μM, respectively for the CHO-AA8 cells. The IC_{50} value computed for the DZR3 cells was 2,400 ± 200 μM. The IC_{50B} value was computed with a 3 parameter fit by setting the $d = Abs_{490min}$ value to 0.

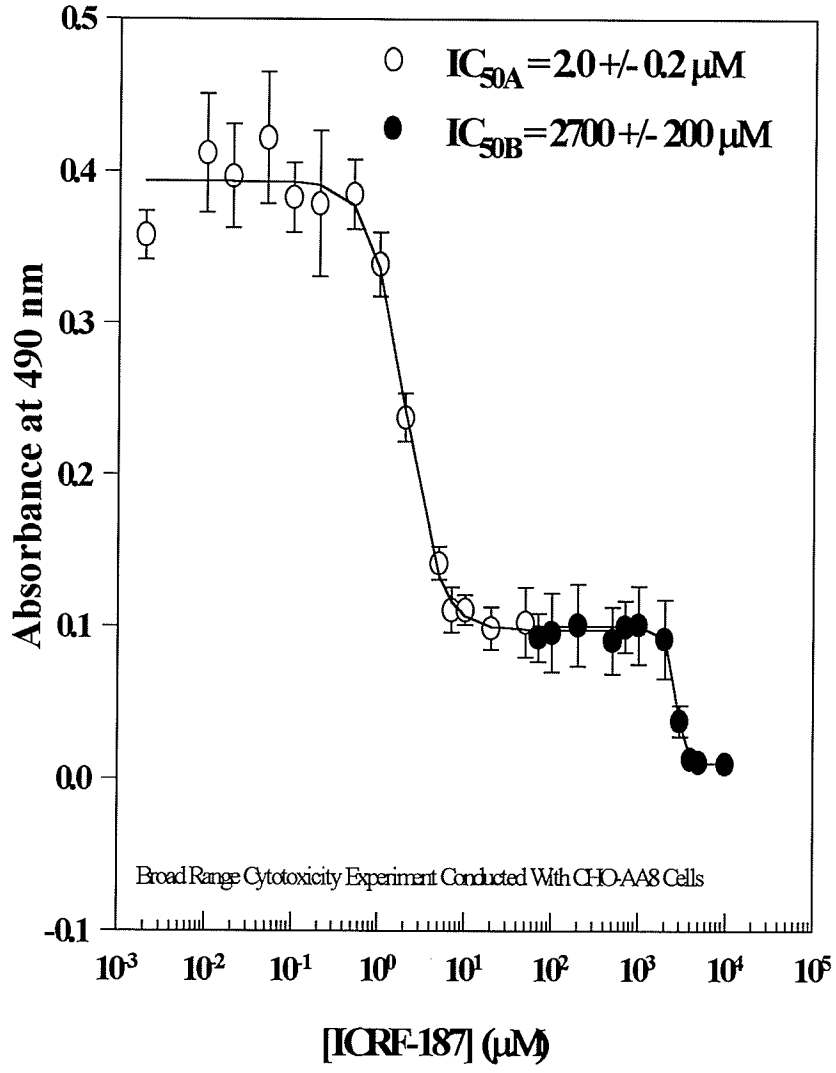
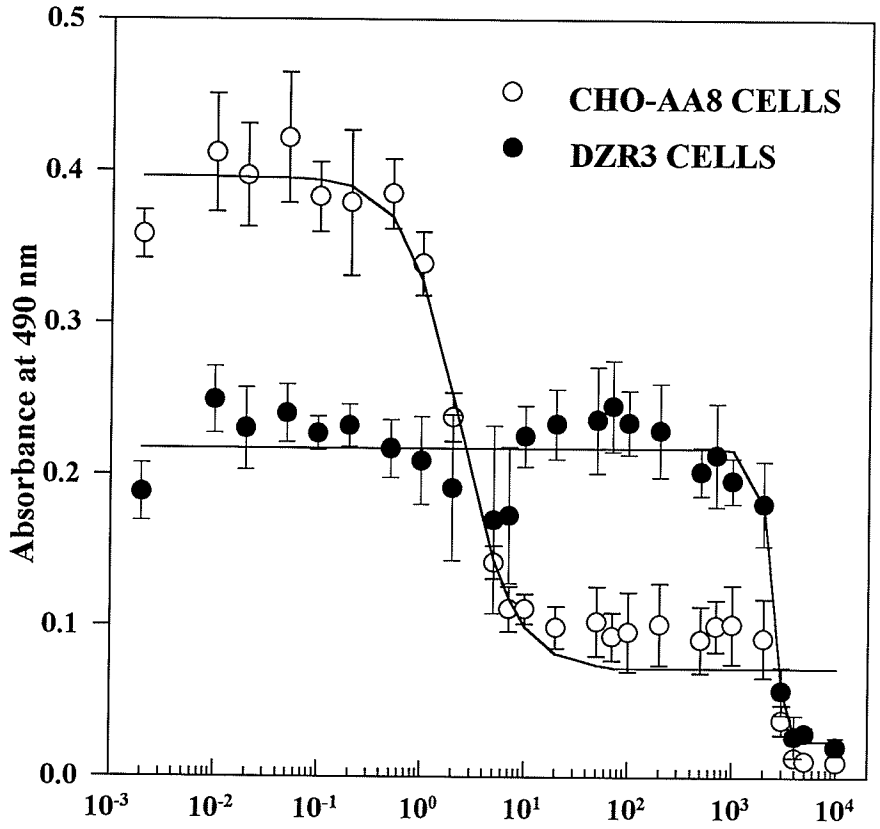


Figure 5.7.1.3.1A MTT Assay of ICRF-187-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells Over A Broad Range Of ICRF-187 Concentrations.



CHO-AA8 IC_{50A} = 2.3 μM **DZR3 IC_{50} = 2400 μM**

CHO-AA8 IC_{50B} = 2200 μM

Figure 5.7.1.3.1B **MTT Assay of ICRF-187-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells And DZR3 Cells.**

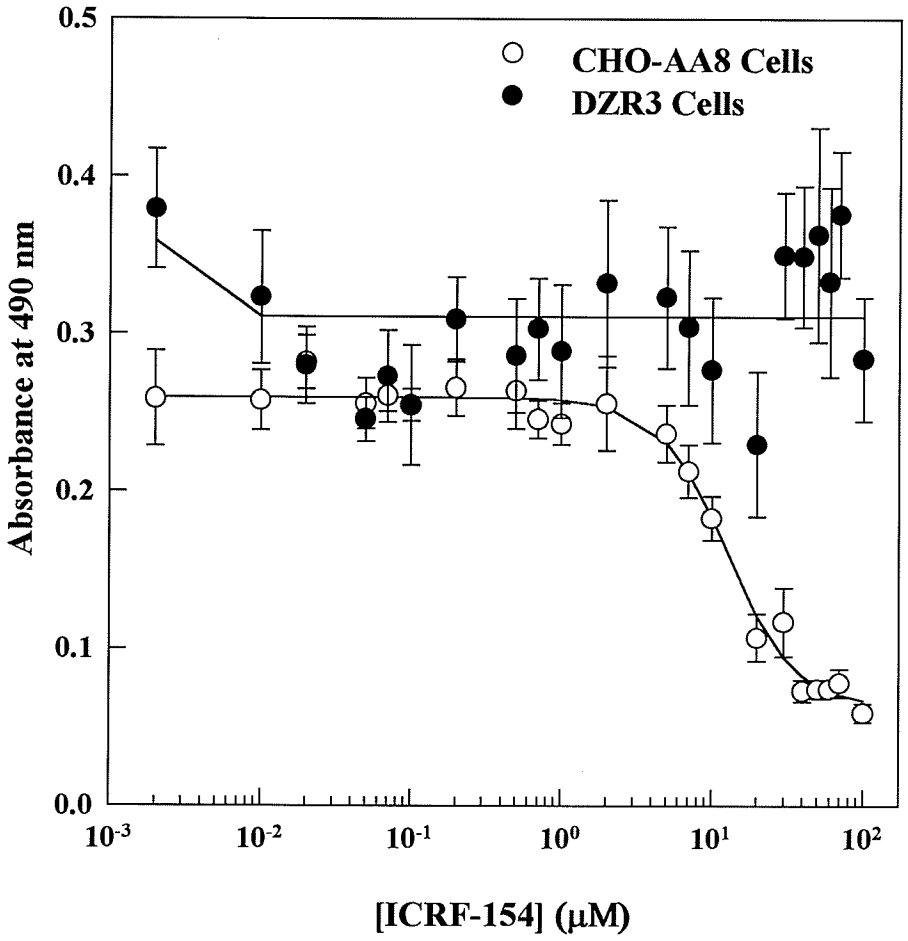


Figure 5.7.1.3.2 **MTT Assay of ICRF-154-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells And ICRF-187-Resistant DZR3 Cells.**

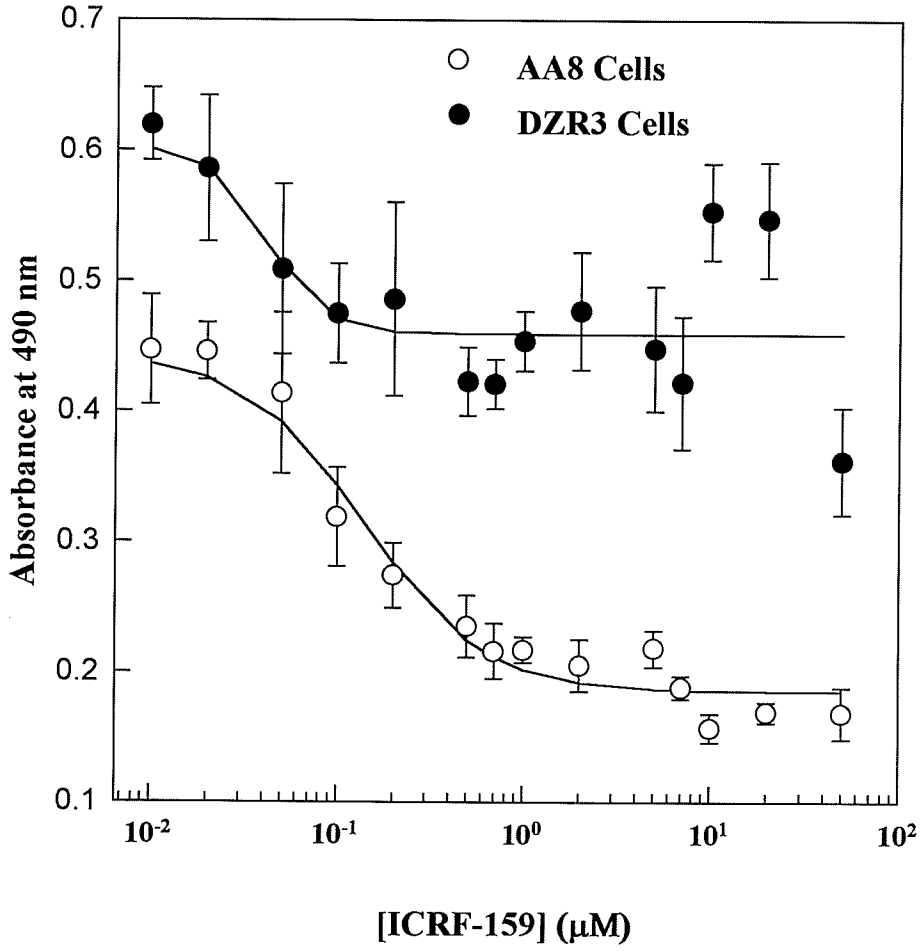
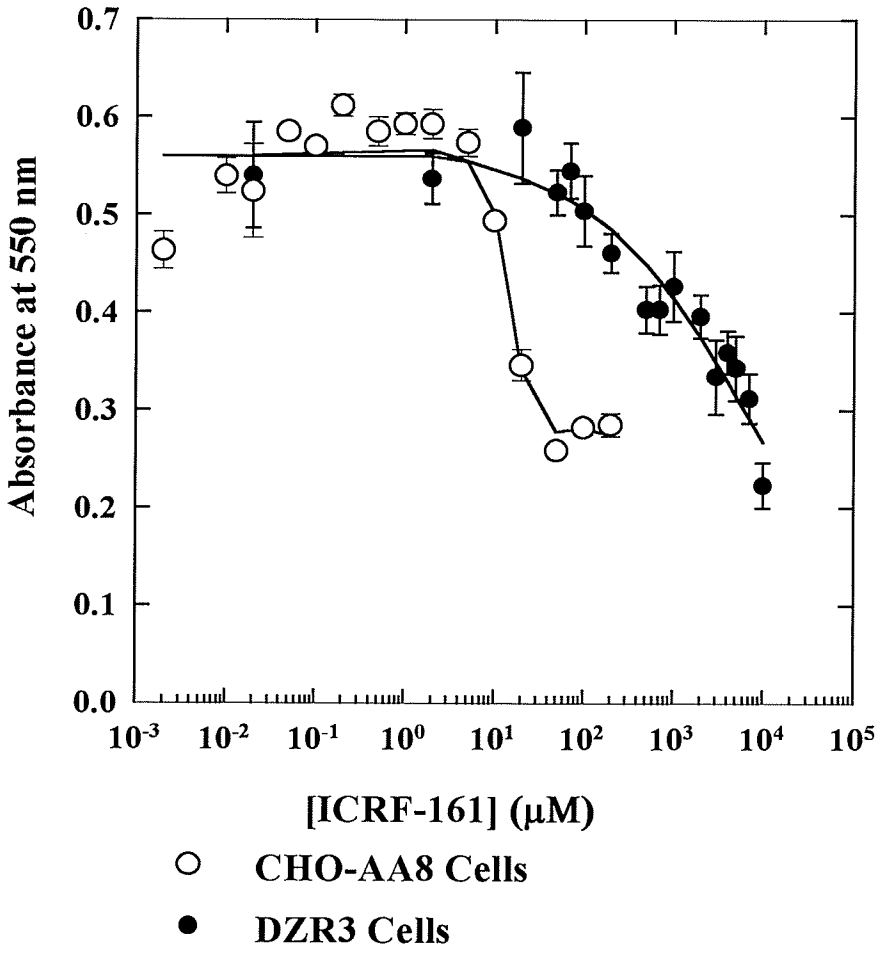


Figure 5.7.1.3.3 **MTT Assay of ICRF-159-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells And ICRF-187-Resistant DZR3 Cells.**



Note: Data points for CHO-AA8 cells were scaled by a factor of 3.8 in order to compensate for low absorbances in the original data.

Figure 5.7.1.3.4 MTT Assay of ICRF-161-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells And ICRF-187-Resistant DZR3 Cells.

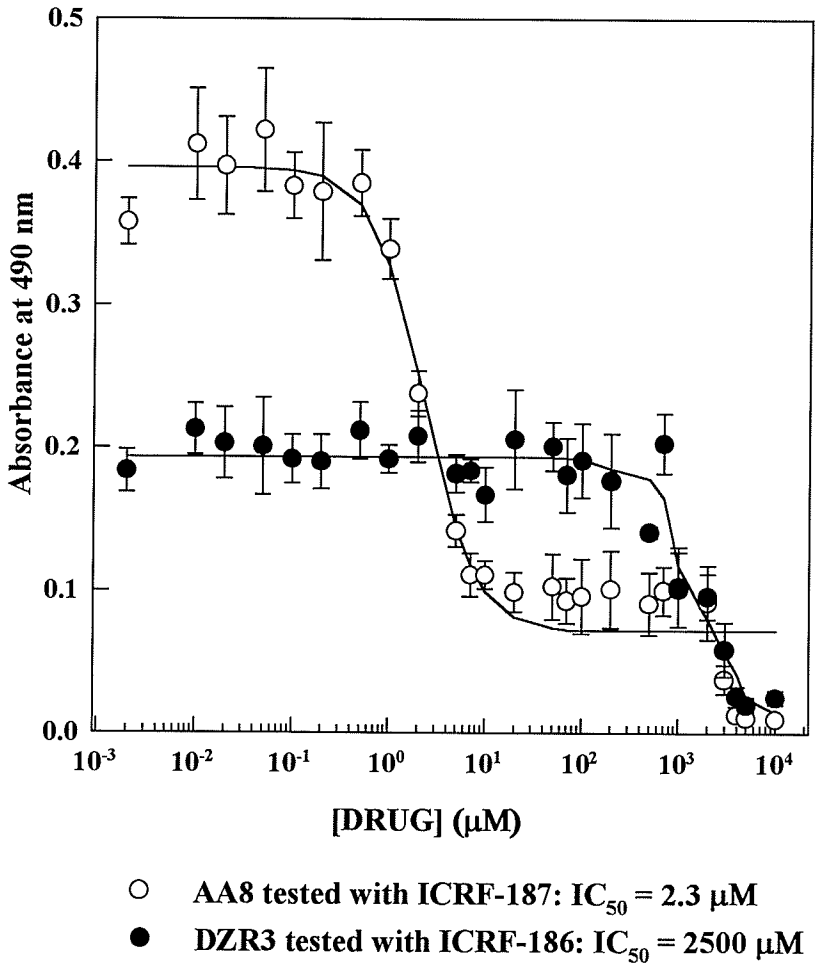


Figure 5.7.1.3.5 **MTT Assay of ICRF-186-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells And ICRF-187-Resistant DZR3 Cells.**

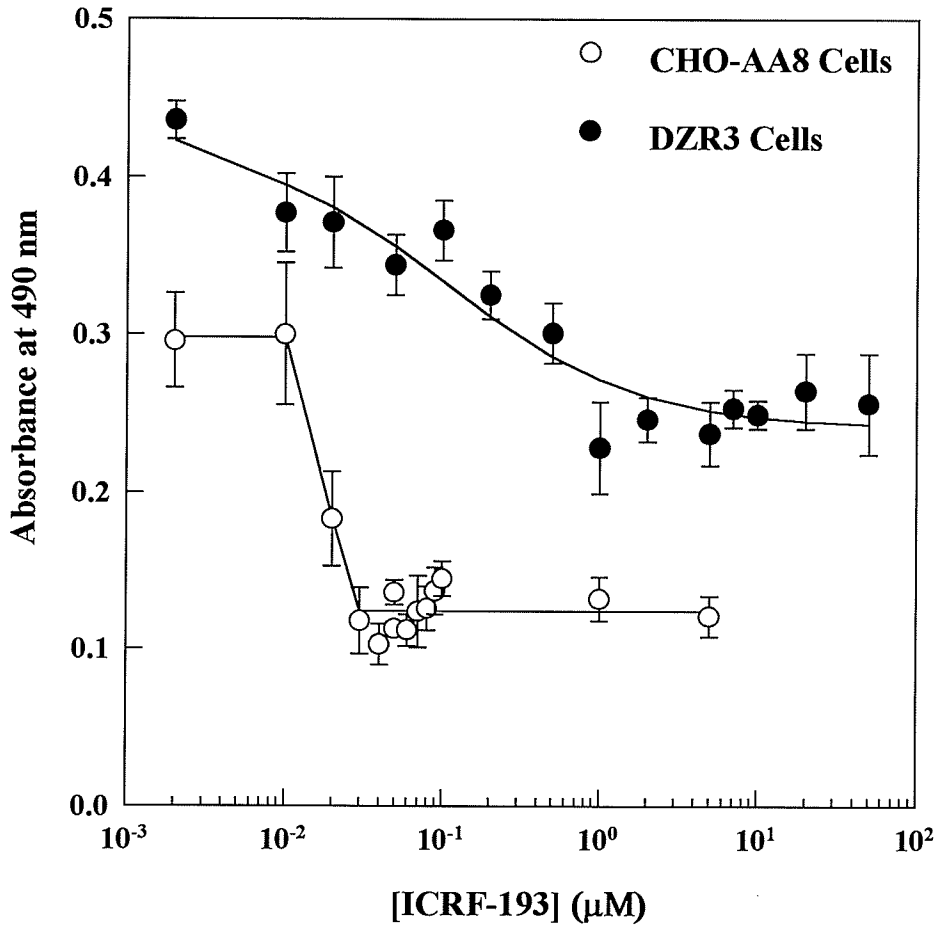


Figure 5.7.1.3.6 **MTT Assay of ICRF-193-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 Cells And ICRF-187-Resistant DZR3 Cells**

Table 5.7.1.3.1 shows all of the IC_{50} and exponential values for the drugs incubated for 48 hours with CHO-AA8 Cells. Table 5.7.1.3.2 shows all of the IC_{50} and exponential values for the drugs incubated for 48 hours with DZR3 Cells.

Table 5.7.1.3.1 IC_{50} Values And Exponential Factors For ICRF Series Bisdioxopiperazines Incubated For 48 Hours With CHO-AA8 Cells

DRUG	IC_{50} Value (μM)	SEM	Exponential Value (b)	SEM
ICRF-154	12.6	1.3	1.9	0.3
ICRF-159	2.7	0.5	2.5	0.7
ICRF-161	14.4	2.5	3.7	1.7
ICRF-186	3.0	0.6	3.5	0.8
ICRF-187	2.7	0.2	2.2	0.2
ICRF-193	0.019	0.002	5.6	3.4

Testing of the ICRF bisdioxopiperazines on the ICRF-187-resistant cells has shown a degree of cross-resistance. Though, the resistance factors of the analogues differ, it is still evident that there is an increase in the amount of ICRF compound required to inhibit 50% of the population. Table 5.7.1.3.3 shows the factor of resistance acquired by the DZR3 cells over the sensitive cells.

Table 5.7.1.3.2 **IC_{50} Values And Exponential Factors For ICRF Series Bisdioxopiperazines Incubated For 48 Hours With DZR3 Cells**

DRUGS	IC_{50} Values (μM)	SEM	Exponential (b) Value	SEM
ICRF-154*	> 500.0	N.D.	N.D.	N.D.
ICRF-159	>250.0	N.D.	N.D.	N.D.
ICRF-161†	8000.0	2000.0	0.49	0.11
ICRF-186	1600.0	400.0	1.6	0.5
ICRF-187	2400.0	200.0	7.4	2.9
ICRF-193	0.11	0.06	0.72	0.26

* The IC_{50} value is defined as being equal to a minimum of five times the maximum drug concentration tested

† $d = Abs_{490min}$ was constrained to 0.

Table 5.7.1.3

A Comparison Of The Resistance Factors Of ICRF Series Bisdioxopiperazines

DRUG	Resistance Factor
ICRF-154	40
ICRF-159	90
ICRF-161	600
ICRF-186	500
ICRF-187	900
ICRF-193	6

The resistance factor is a comparison of the degree of drug resistance of the wildtype cells and the resistance cells to a particular agent. It is calculated by dividing the IC_{50} of the drug tested on the resistant cell line by that of the IC_{50} of the same drug tested on the wildtype cells, *i.e.*,

$$\textit{Resistance Factor} = IC_{50}\ DZR3 / IC_{50}\ CHO\text{-}AA8$$

5.7.1.4 DISCUSSION

The selection of an ICRF-187-resistant cell line has resulted in a clone which has acquired resistance not only to the ICRF-187 compound, but to a number of ICRF-187 analogues as well. Though the degree of cellular resistance to he toxicity of these analogues differs, these cells have shown a degree of increased resistance to all of the compounds tested. The DZR3 cells had acquired some resistance to the most toxic of the compounds tested, ICRF-193, but only a marginal resistance as compared with that acquired over the other less toxic analogues. The resistance factor of ICRF-193 was only a factor of 6 greater in the resistant cells than in the sensitive cells. The resistance factor of the DZR3 cells over the CHO-AA8 cells in the case of ICRF-187 was 900. Though the differences in the IC_{50} values for the ICRF-187 and ICRF-186 analogues were very similar in the CHO-AA8 cells, they were substantially different in the DZR3 cells. Resistance to ICRF-186 was only 500 fold greater in the resistant cells. This suggests that there is a difference in the degree of protective capacity aquired against the toxic effects of the selecting agent, ICRF-187 over that of ICRF-186, its *(-)-(R)* isomer. Perhaps where

there is development of a protective mechanism to deal with topoisomerase II inhibitors, there is a marked difference in the isomers and how both the sensitive and resistant cells deal with them. The resistance factor of the DZR3 cells is 600 for ICRF-161. This is almost the same as the value for ICRF-186. This would indicate that the alterations made by the resistant cells were specific in dealing with the toxicities inflicted by ICRF-187, but the cells were less accommodating in their resistance to the other analogues. The IC_{50} for ICRF-154 incubated for 48 hours with DZR3 ICRF-187-resistant cells was defined as being equal to a minimum of five times the minimum concentration tested *i.e.* 500 μM. Assuming the IC_{50} for the ICRF-154 incubated with DZR3 cells for 48 hours is 500 μM, the resistance factor would be 40 fold over the resistance to ICRF-154 of the CHO-AA8 cells. Similarly, for ICRF-159, the IC_{50} was estimated as being a minimum of five times the maximum drug concentration tested, *i.e.* 250 μM. Thus, the resistance factor over the wildtype is 90 fold.

These data correlate well with data presented by White and Creighton, (1976), who found that there is consistent development of cross resistance only to structural analogues of the selecting agent, which in the case of their experiments was ICRF-159. They also found that there was a consistent, but limited acquisition of cross-resistance to anthracyclines tested. This is discussed below.

5.7.2 Cross-Resistance To Anthracyclines

5.7.2.1 INTRODUCTION

Anthracyclines are widely used clinical cancer chemotherapeutic agents. Drugs such as doxorubicin and actinomycin D are known to be intercalators of DNA (D'Arpa and Liu, 1989) as well as putative inhibitors of cardiac Na^+/K^+-ATPase (Nogrady, 1988). An increasing amount of evidence is indicative of the probability that toxicity may be due to a Fenton-type iron-dependent free radical stress (Halliwell and Gutteridge, 1985). *In vitro* studies suggest that the damage of cellular components that is mediated by doxorubicin is dependent upon iron. It is the multifaceted capacity to damage cells that makes the anthracyclines like doxorubicin effective chemotherapy agents. While most cells are able normal cells in the body are able to protect themselves from the damaging effects of these agents, the heart lacks the capacity to do so, and thus the use of doxorubicin is limited by the risk of dose-limiting cardiotoxicity (Gianni *et al.*, 1983; Halliwell and Gutteridge, 1985).

Recently, anthracyclines have been shown to act on topoisomerase II. A study involving several anthracyclines demonstrated a strong correlation between potency of intercalation and formation of cleavable complexes. It has been suggested that during the normal unwinding of the DNA generated by topoisomerase, the intercalation of the DNA by anthracyclines causes the normally apposed, broken DNA strands to become misaligned or disjointed within the cleavable complex (D'Arpa and Liu, 1989).

Chapter 5 The ICRF-187-Resistant Cell Line, DZR3

The experiments conducted with the DZR3 ICRF-187-resistant cells were performed in order to determine whether there was any detectable change in the degree of anthracycline-induced insult suffered by the resistant cells in comparison with the sensitive parental line. Seeking potential cross-resistance to anthracyclines in a bisdioxopiperazine-resistant cell line, will elucidate the true target, or at least the differences between the targets of each drug. If cross-resistance were found this might indicate similar mechanisms or targets. If no cross-resistance is detected, it is indicative of clearly different mechanisms of insult by the two drugs. These experiments were conducted with only the resistant cells in this series since Chapter 4 is devoted to the interactions of anthracyclines and bisdioxopiperazines and their metabolic products.

Since the effects of most of the anthracyclines tested were similar, and the only significant differences in the toxicity of anthracyclines was seen in doxorubicin and idarubicin, whose IC_{50} values differed by a factor of 10, only these two drugs were tested for cross-resistance and additive toxicity or protection from cytotoxicity mediated by addition of 300 μM ICRF-187 to one set of plates. The concentration of ICRF-187 was selected as 300 μM since it was high enough to be potentially cytotoxic with the addition of doxorubicin, and yet not mediate any cytotoxicity on its own since the cells were capable of relatively normal growth in 1 mM ICRF-187. Experiments conducted with idarubicin and doxorubicin, both in the presence and absence of 100 μM ICRF-187 showed no difference between the curves with ICRF-187 and those without. (Data not shown). For this reason, a higher concentration was used. Also, at a high ICRF-187

concentration, any protective effect mediated by ICRF-187 would be noticeable.

5.7.2.2 METHODS AND MATERIALS

5.7.2.2.1 Seeding Of The Microtitre Plates With Cells

DZR3 cells were grown to near-confluence in a canted 25 cm^2 T-flask in αMEMcontaining 10% (v/v) calf serum. The media was removed, the cells were washed with Dulbecco's PBS and then trypsinized. After quenching and centrifugation, the cells were resuspended in 10 mL of cell culture medium and counted six times. The average was taken and the cells were diluted appropriately to give a cell suspension of 10,000 cells mL^{-1}. Four microtitre plates were seeded with 100 μL volumes of this suspension to give well concentrations of 1,000 cells per well. The cells were grown in darkness in the incubator overnight at 37°C in an atmosphere of 5% CO_2 and 95% air (v/v).

5.7.2.2.2 Preparation Of The Drugs

After the 24 hour anchoring period, 1 mM stock solutions of doxorubicin and idarubicin were removed from refrigeration and were allowed to warm to room temperature. The doxorubicin solution was diluted 10:1 serially in 150 mM NaCl solution to 1 mL volumes of 100 μM, 10 μM, 1 μM, and 0.1 μM concentrations. The idarubicin was diluted to 1 mL volumes of 100 μM, 10 μM, 1 μM, 0.1 μM and 0.01 μM concentrations. A sample of ICRF-187 was weighed out and dissolved in αMEM to a concentration of 1,200 μM, and filer sterilized by syringe filtration through a 0.2 μm filter. The anthracyclines were not filtered since much of the drug would adhere to the

filter and be lost. Also, its is not considered a source of contamination since it is an extremely toxic antibiotic.

5.7.2.2.3 Delivery Of the Drugs To The Microtitre Plates

Delivery of doxorubicin was made to two microtitre plates from substock solutions of 0.1μM, 1μM, 10 μM, and 100 μM solutions. Volumes added were no less than 10 μL and no greater than 50 μL. Any differences were made up to 50 μL with sterile 150 mM NaCl solution to give a well volume of 150 μL. The drug volumes delivered were to give a final range of concentration spanning from 0.01-50 μM plus a 0 μM control. One plate received 50 μL of αMEM to give a total well volume of 200 μL and the correct final doxorubicin concentrations. The other plate received 50 μL of 1,200 μM ICRF-187. This gave a total well volume of 200 μL with the correct final doxorubicin concentrations as well as 300 μM ICRF-187. Thus, both plates contained the same range of doxorubicin concentrations, while one contained 300 μM ICRF-187 and the other did not. The same was done in the idarubicin experiment, except that the range of idarubicin concentrations measured for cytotoxicity was 0.001-5.0 μM as well as the 0 μM control.

Drugs were added using an 8 x 200 Gilson multichannel pipettor. The "make up" volumes of NaCl solution were added first to reduce the toxicity of the concentrated anthracyclines. The anthracyclines were added next. The αMEM or the αMEM containing the 1,200 μM ICRF-187 was added last and mixing of drugs in the wells was facilitated by mixing thoroughly after the final addition. The plates were allowed to incubate in the above mentioned conditions for 48 hours.

5.7.2.2.4 *End-point Detection*

After the 48 hour incubation of the cells with drug, 20 μL of 2.5 mg mL^{-1} MTT solution was added to the wells containing cells and allowed to incubate for another 4 hours. After this incubation, the medium was carefully aspirated off with an 18 gauge needle, without disturbing the cell layer, 100 μL of spectral grade DMSO was added to all the wells including those defined as blanks. After the formazan crystals were dissolved in the DMSO, the plates were read at λ= 490 nm minus λ = 650 nm.

5.7.2.3 RESULTS

Figure 5.7.2.3.1 illustrates the effects of a range of doxorubicin concentrations with, and without 300 μM ICRF-187 on DZR3 ICRF-187-resistant cells. Figure 5.7.2.3.2 illustrates the effects of a range of idarubicin concentrations, with and without 300 μM ICRF-187 on DZR3, the ICRF-187-resistant cells.Table 5.7.2.3.1 lists the exponential factor (*b*) and IC_{50} values for the doxorubicin ± 300 μM ICRF-187 and idarubicin ± 300 μM ICRF-187 experiments.

Table 5.7.2.3.1

Exponential Factors And 50% Inhibitory Concentrations For Doxorubicin And Idarubicin In The Presence And Absence Of 300 μM ICRF-187 Incubated For 48 Hours With DZR3 ICRF-187-Resistant Cells

Drug Combination	IC_{50} Value (μM)	SEM	Exponential Factor	SEM
DOX - 300 μM ICRF-187	3.3	1.7	0.78	0.17
DOX + 300 μM ICRF-187	3.3	1.6	0.78	0.17
IDA - 300 μM ICRF-187*	0.10	0.03	0.64	0.12
IDA + 300 μM ICRF-187*	0.11	0.05	0.58	0.05

The IC_{50} values were computed using the equation,

$$Abs_{490obs} = (a-d)/(1+(x/c)^b)+d$$

where $a = Abs_{490min}$, $d = Abs_{490min}$, c = the IC_{50} value computed in the fit, b = the exponential factor, which give the rate of increase of cytotoxicity as a function of the increase in drug dose, and x values are experimental drug doses used in the experiment. These are selected from the column containing the drug concentration data in the Sigma plot program. The drugs marked with the * symbol were calculated in a 3 parameter fit where the $d = y(min)$ values were set to 0, and the b and c values were computed from the original a value and the best guesses for b and c from the 2 parameter fit.

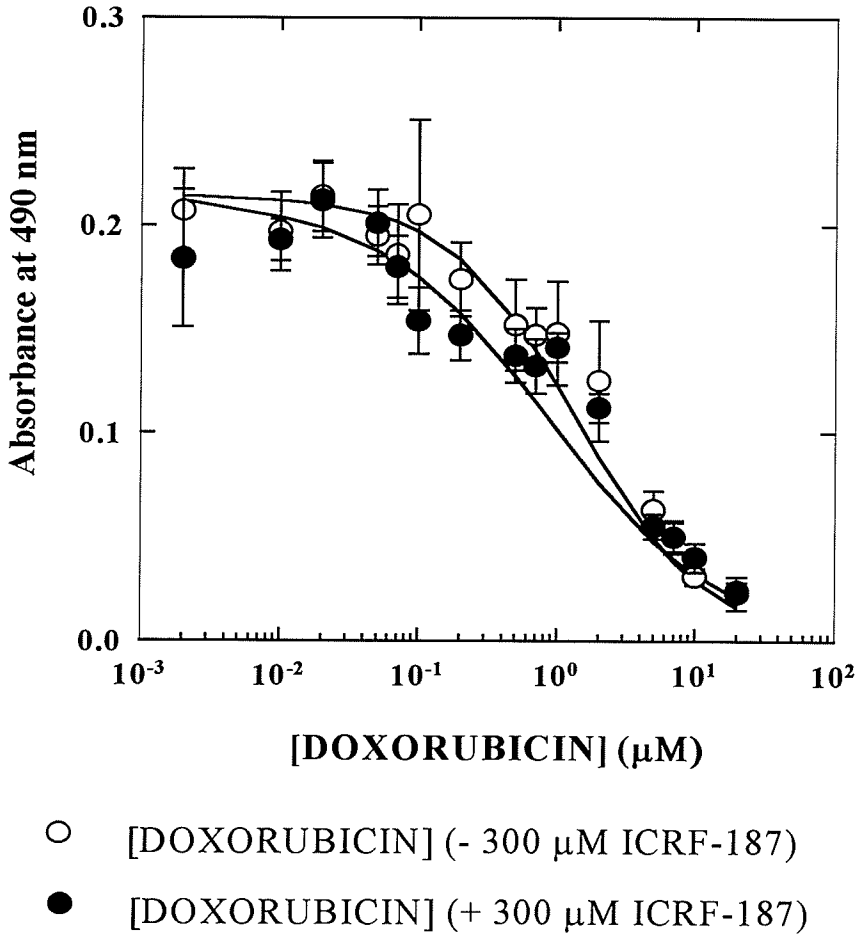


Figure 5.7.2.3.1 **MTT Assay Of Doxorubicin-mediated Cytotoxicity On ICRF-187-Resistant DZR3 Cells Incubated For 48 Hours In The Presence And Absence Of 300 μM ICRF-187**

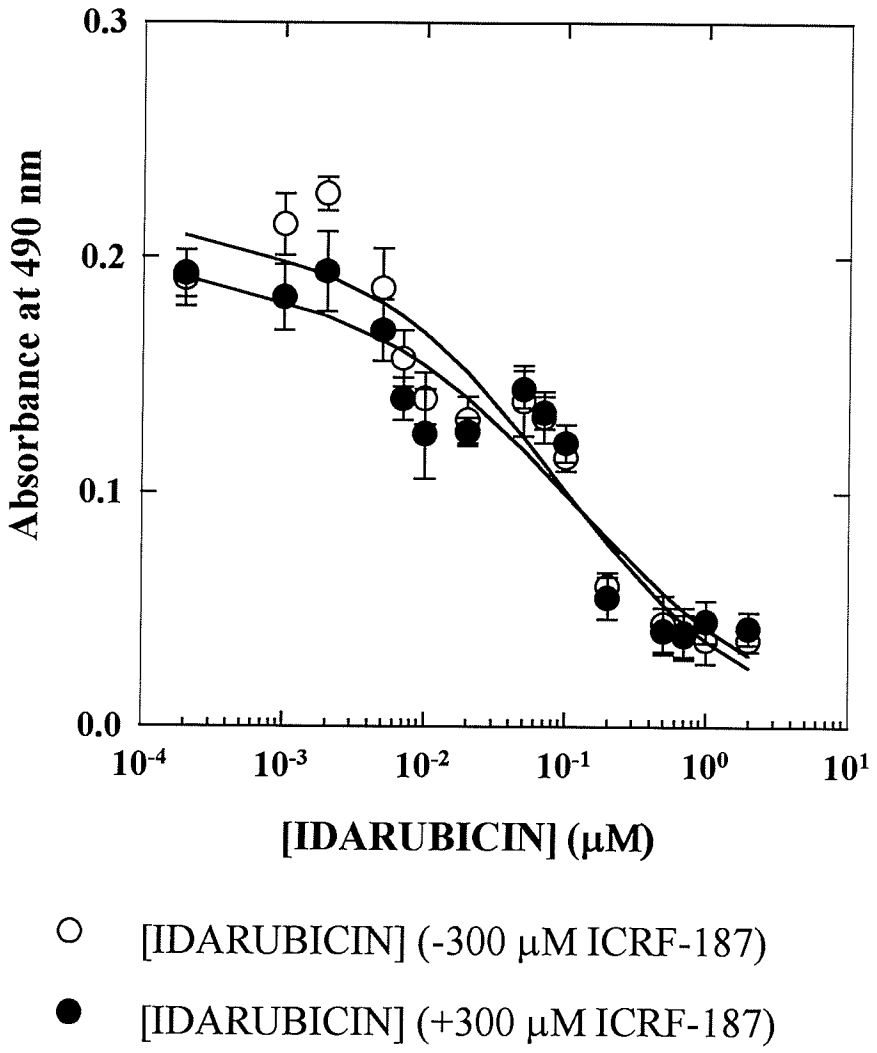


Figure 5.7.2.3.2 **MTT Assay Of Idarubicin-mediated Cytotoxicity On ICRF-187-Resistant DZR3 Cells Incubated For 48 Hours In The Presence And Absence Of 300 μM ICRF-187**

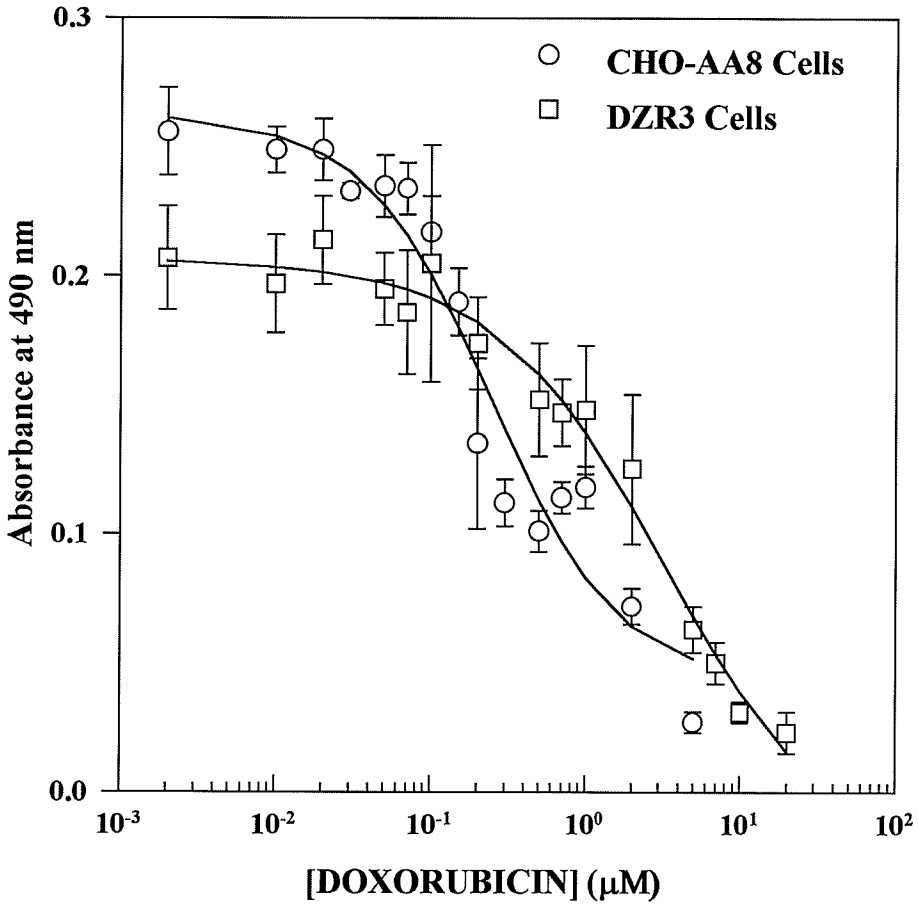


Figure 5.7.2.3.3 Comparison Of Resistance Of CHO-AA8 And DZR3 Cells To Doxorubicin After 48 Hours Incubation

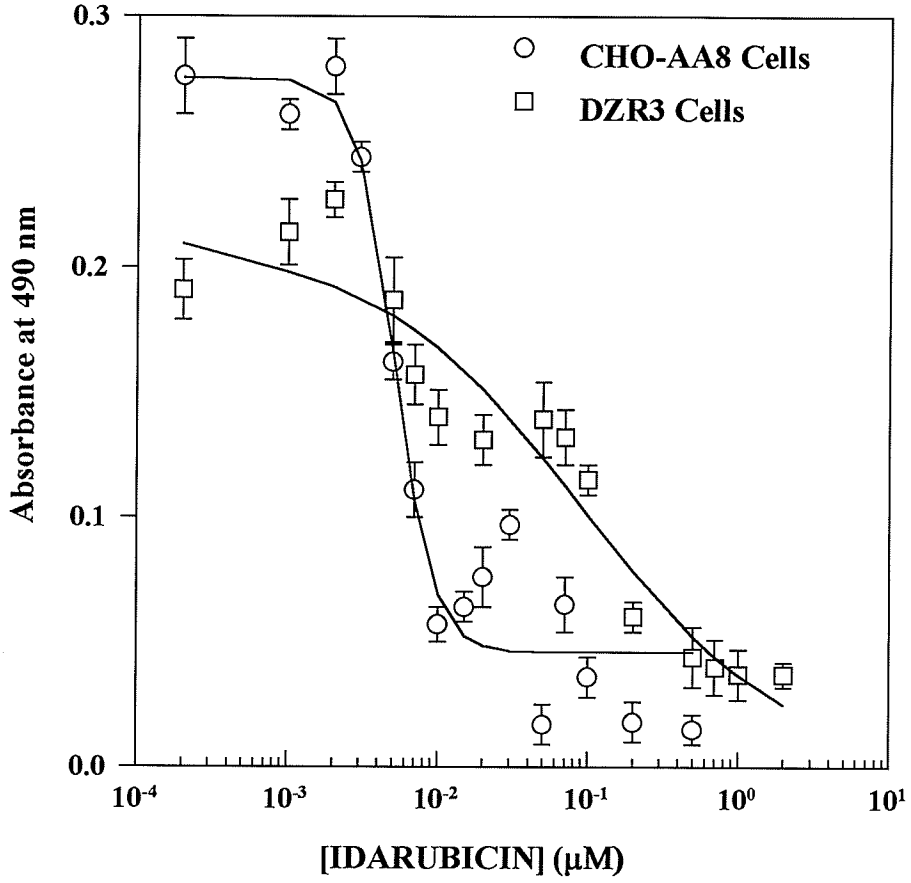


Figure 5.7.2.3.4 Comparison Of Resistance Of CHO-AA8 And DZR3 Cells To Idarubicin After 48 Hours Incubation

5.7.2.4 DISCUSSION

As is shown in Table 5.7.2.3.1, there is virtually no difference in the IC_{50} values computed in the doxorubicin ± 300 μM ICRF-187. However, toxicity mediated by doxorubicin after the customary 48 hour incubation with DZR3 cells showed values different from those computed for CHO-AA8 cells. Where the IC_{50} values for doxorubicin and idarubicin incubated with CHO-AA8 cells are 0.25 ± 0.08 μM and 0.0035 ± 0.0008 μM, respectively, the values seen for the DZR3 cells are 3.3 ± 1.7 μM and 0.10 ± 0.003 μM. For doxorubicin and idarubicin, these values represent resistance factors of 13.2 and 28.5, respectively. (Figures 5.7.2.3.3 and 5.7.2.3.4) The addition of 300 μM had little consequence in terms of adding to or ameliorating the effects of doxorubicin. The experiments conducted with idarubicin in the presence or absence of 300 μM ICRF-187 were essentially the same. Though, the cytotoxicity of idarubicin is greater than that of doxorubicin, the trend is essentially the same. The difference between the IC_{50} values of the idarubicin experiment without ICRF-187 as compared to the value with the drug, was 0.01 μM however, the errors in each of these inhibitory concentrations were 0.03 and 0.05 μM, respectively. This suggests that differences between these values is not significant.

Overall, the resistant cells are able to protect themselves from the cytotoxic effects of the two representative anthracyclines better than CHO-AA8 cells. Doxorubicin showed a resistance factor of 13.2 and idarubicin resistance was shown to be 28.5 fold. ICRF-187 caused no additional damage and offered no protection from the effects of the anthracyclines.

5.7.3 Cross-Resistance Among Other Antineoplastic Agents

5.7.3.1 INTRODUCTION

In addition to the bisdioxopiperazines and the anthracyclines which are known to be associated in multidrug resistance (MDR), a number of other drugs were tested in order to determine whether any other cross-resistance in the DZR3 cells. Some of the agents tested in this project are well known as being associated with MDR. These include etoposide (from the epipodophyllotoxin family), and vinblastine (from the vinca alkaloid family). Those drugs tested which are not associated with the MDR phenomenon are alkylating agents such as cyclophosphamide, antimetabolites such as 5-fluorouracil and methotrexate, and miscellaneous agents such as cisplatin, bleomycin and hydroxyurea.

Etoposide does not show any evidence of binding DNA however, addition of this compound to cells was found to stimulate the formation of topoisomerase II-DNA cleavable complexes, followed by cell death. The formation of topoisomerase II-DNA cleavable complexes after the addition of etoposide was found to be true not only in cultured cells, but in purified mammalian topoisomerase II (Bodley and Liu, 1988). Again, if the compound does not bind DNA, but binds the DNA-topoisomerase II complex, perhaps alteration of DZR3 topoisomerase in response to stress with ICRF-187 has allowed the topoisomerase to be altered sufficiently to be resistant not only to ICRF-187, but to etoposide as well.

The alkylating agents were developed as antitumor agents which are able to covalently bind two strands of DNA by virtue of their carbonium or other electrophilic

groups, and thereby interfere with transcription or other replication processes. Since these replication processes occur with more frequency in rapidly dividing malignant cells than in normal cells, these agents can effectively reduce or eliminate tumors. One particular alkylating agent, cyclophosphamide, was synthesized in the hope of exploiting phophoraminidase enzyme, found in high concentrations in tumor cells. It is inactive until it is metabolized in the liver. Though it has side effects, it is an effective agent in treating a number of carcinomas and lymphomas (Nogrady, 1988).

Unlike the non-alkylating agents, where high levels of resistance (greater than 2-3 log units) can be achieved, selecting cells for resistance to alkylating agents is very difficult. Usually, stable resistance is only 2-5 fold higher than that in sensitive cells. Though, this is unfortunate from the research perspective, it does hold promise in the battle against MDR, where patients often develop resistance against other classes of antitumor drugs and succumb after a short remission. Mechanisms of resistance to alkylating agents differ among cell lines and among agents as well. This agrees with the fact that there is little cross-resistance among the different agents. Cyclophosphamide is believed to cause resistance by increasing the amount of cellular aldehyde dehydrogenase. Alkylating agents commonly have multiple forms of resistance within the same clone. This is exemplified by cisplatin which acts to increase cell resistance by implementing transport reduction, increases in levels of both glutathione transferase and protein-sulfhydryl (Frei *et al.*,1988).

The vinca alkaloids are a class of cytostatic agents which are able to interfere with the mitosis of cells. More specifically, they are able to bind the αβ dimers of the microtubules which pull daughter cells apart during cytokinesis, and effectively inhibit their incorporation into the growing microtubules. Vinblastine is one such vinca alkaloid and is effective in treating various leukemias as well as Hodgkin's lymphoma however, vinca alkaloids have also shown high levels of neutrotoxicity (Nogrady, 1988).

Examination of the morphology of cells made resistant to vinblastine shows an increase in the amount of cellular vacuoles in comparisons to the control cells. There was uneven staining in the nuclear stains (Beck *et al.*, 1986). The cells took on irregular shapes and showed increases in ebbing and vacuolization. The nature of these vacuoles is not yet clear. However, they somehow affect the ability of the cells to withstand high levels of the alkaloid. Addition of verapamil, a calcium channel blocker able to reduce MDR in drug resistant cells, results in enhanced verapamil toxicity in the vinblastine-resistant cells (Beck *et al.*, 1986). Cellular drug accumulation experiments measuring the combined effects of influx, intracellular drug binding, and afflux using radiolabeled vinblastine showed a 2-fold decrease in the accumulation of drugs within an MDR tumor cell as compared to a sensitive cell line (Fojo *et al.*, 1985). Most MDR cells studied at the point of publication of Lum's work in 1993, showed enhanced afflux rather than a decrease in influx of compounds (Dalmark and Storm, 1981). These MDR phenomena are most consistently associated with the increase in the increased synthesis of the 170-kDa plasma membrane-associated P-glycoprotein (Kartner *et al.*, 1985). Additional work in the

area of the increased vacuolization has indeed shown a role for vacoules and secretory vesicle in the drug afflux mechanism of resistant cells. Dietel *et al.*, (1992) suggest that MDR related drugs are packaged into these perinuclear vesicles which are bound to tubulin and transported out of the cell.

Another group of antitumor agents which also interferes with the transcription of DNA are the platinum complexes. The *cis*-dichloroammine-platinum (II) complex, also known as cisplatin, is very active against testicular tumors, epidermoid carcinomas and ovarian tumors. It interacts very strongly with DNA by intrastrand binding (not the same as cross-linking) to oligoguanine sequences, unwinding the duplex and reducing the length of the DNA molecule (Nogrady, 1988).

In vitro exposure of TR170 ovarian carcinoma cells to 6 intermittent 24 hour treatments with an IC_{90} concentration of *cis*-dichloroammine-platinum (II) (cisplatin) resulted in a 2-fold order of resistance after 6 treatments. There were no significant differences between these cells and the wildtype cells in terms of growth characteristics or karyotypic alterations. Glutathione and related enzymes found in the same levels in both resistant and sensitive cells as were levels of superoxide dismutase. Resistance to cisplatin in the drug selected clones was shown to be associated with decreased accumulation of the drug. Decreased uptake of the drug as well as a decrease in the removal of certain Platinum-DNA adducts indicates a possible increase of tolerance for the DNA damage induced by the cisplatin (Hill *et al.*,1992).

Work by Saburi *et al.*, (1989) with cisplatin-resistant CHO cells selected over a period of 5 months of exposure to cisplatin showed the cisplatin-mediated damage was in the form of interstrand DNA-DNA and DNA-protein cross-links. Cisplatin is not believed to be a substrate for P-glycoprotein. Of the three classes of glutathione-S-transferase, GST-μ, GST-α, and GST-π, only the latter was found in higher concentrations in these cells. It is their feeling that the acquisition of cisplatin resistance is associated with increased expression of increased levels of GST-π. Evidence suggests however, that the acquisition of cisplatin resistance is not restricted to GST-π alone; the resistance to cisplatin is multifaceted phenomenon, which includes increase sulfhydryl protein activity, plasma membranes activities, as well as increased DNA lesion repair (Saburi *et al.*, 1989).

Antitumor activity against breast cancer as well as some intestinal cancer carcinomas has been found in the antimetabolite 5-fluorouracil which irreversibly complexes with tetrahydrofolate and the enzyme thymidilate synthase. Recovery can occur only through the synthesis of new enzyme (Nogrady *et al.*, 1988).

Another antimetabolite which inhibits the synthesis of thymidilate synthase by irreversible blocking of dihydrofolate reductase is methotrexate. Methotrexate is used to treat acute childhood leukemia and choriocarcinoma.and treatment usually results in 30-50% and better than 70% remission rates, respectively. Though it is an effective antitumor drug, it does not distinguish between the dihydrofolate reductase of the healthy cells and the cancer cells, and has severe side effects (Nogrady *et al.*, 1988).

Among the cytostatic agents is a very complex group of glycopeptides known as the bleomycins. These compounds have a large repertoire of activities, including scission and breakage of viral, bacterial, and mammalian DNA. They are able to inhibit DNA ligase as well. Thus, bleomycin is able to interfere with not only the transcription, but the replication of nucleic acids. These agents are particularly effecting in treating squamous-cell carcinoma of the neck and head since there are no bleomycin-degrading enzymes present (Nogrady, 1988).

Hydroxyurea is classified as a nucleic acid base analogue which inhibits the function of ribonucleotide reductase, and is able inhibit DNA synthesis (Timson, 1975). It is of interest as an antineoplastic however, its mechanism of inhibition have not been established. It is known that hydroxyurea may inhibit sterically, and that it somehow results in the one-electron reduction of tyrosyl radical (Sahlin *et al.*, 1982). Whether this involves direct enzyme binding or a hydrogen atom transfer is not yet known .

5.7.3.2 METHODS AND MATERIALS

5.7.3.2.1 Seeding Of The Microtitre Plates With Cells

CHO-AA8 and DZR3 cells were grown in 25 cm^2 T-flasks to near confluence. The c\flasks were washed with Dulbecco's PBS, trypsinized, and centrifuged. The supernatant was poured off and the pellet was resuspended in 10 mL of αMEM. The two cultures were counted separately and diluted to the cell number required for the experiment. The CHO-AA8 cells were diluted to 20,000 cells mL^{-1} and seeded in a 100 μL volume into

a 96-well microtitre plate to give a total well concentration of 2,000 cells per well. The DZR3 cells were diluted to 50,000 cells mL^{-1} and seeded in a 100 μL volume to give a total well concentration of 5,000 cells per well. Usually two plates of cells were seeded per drug per cell line thus, on average, each experiment required four 96-well plates.

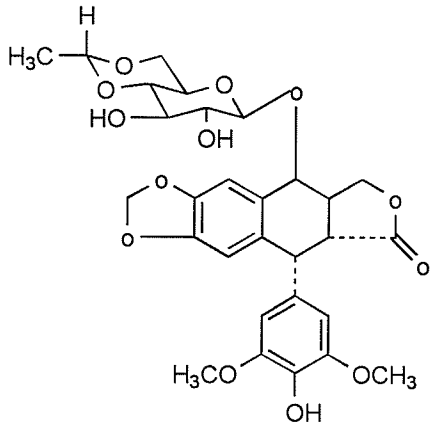
5.7.3.2.2 Preparation of Drugs

Where drugs were readily soluble in αMEM, they were dissolved in concentrations as high as 20 mM. In certain cases, 20 to 30 minutes of sonication were required to completely dissolve a drug. These included cyclophosphamide, 5-fluorouracil, and hydroxyurea. Where drugs were soluble in cell culture medium to a slightly lesser degree *i.e.* 2 mM, or if they were known to be highly cytotoxic, they were dissolved in αMEM to the limit of their solubility, or to the limit required to deliver a range of drug concentrations covering the cytotoxic concentration range of the drug. The drugs dissolved in αMEM at lower concentrations included bleomycin, doxorubicin, methotrexate, and cisplatin. DMSO was used for the drugs that proved to be extremely difficult to dissolve in an aqueous environment. These included etoposide and vinblastine. These were known to be extremely toxic and were prepared in sufficient concentration to facilitate the delivery of adequate concentrations of drug in 1.0 μL DMSO to give the correct final drug concentration in 200 μL.

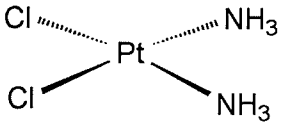
5.7.3.3 RESULTS

The experimental results for the cross-resistance experiments are illustrated in the plots shown on the following pages and the effects of the drugs, whose effects were measured by 48 hour incubations with both CHO-AA8 and DZR3 cells, are listed in Tables 5.7.3.3.1 and 5.7.3.3.2. The former lists the IC_{50} values and the exponential values for the cytotoxicity experiments conducted with CHO-AA8 cells, while the latter lists the same information for the experiments conducted with DZR3 cells. Figures 5.7.3.3.1 and 5.7.3.3.2 illustrate the structures of the agents tested, while Figures 5.7.3.3.3 through 5.7.3.3.10 illustrate the cytotoxicity plots of these drugs, on CHO-AA8 and DZR3 cells.

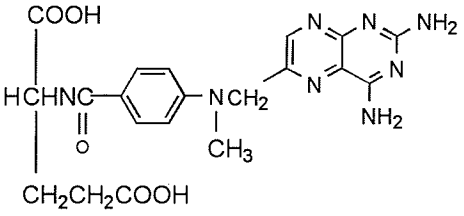
STRUCTURE OF DRUGS USED TO TEST FOR MULTIDRUG RESISTANCE IN ICRF-187-RESISTANT DZR3 CELLS



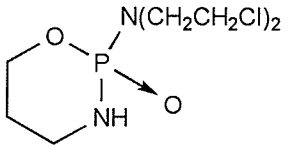
ETOPOSIDE
Molar Mass: 588.6 g/mol
SOLUBILITY in DMSO ~ 20 mM



CISPLATIN
Molar Mass: 300.5 g/mol
SOLUBILITY in DMSO ~1 mM

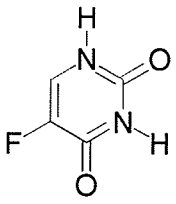


METHOTREXATE
Molar Mass: 440.43 g/mol
SOLUBILITY in DMSO ~ 10 mM

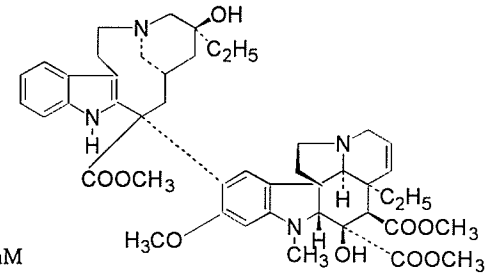


CYCLOPHOSPHAMIDE
Molar Mass: 261.10 g/mol
SOLUBILITY in αMEM ~ 20 mM

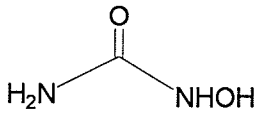
Figure 5.7.3.3.1 Illustration Of The Chemical Structure Of Drugs Incubated With CHO-AA8 And DZR3 For 48 Hours To Test For Cross-Resistance



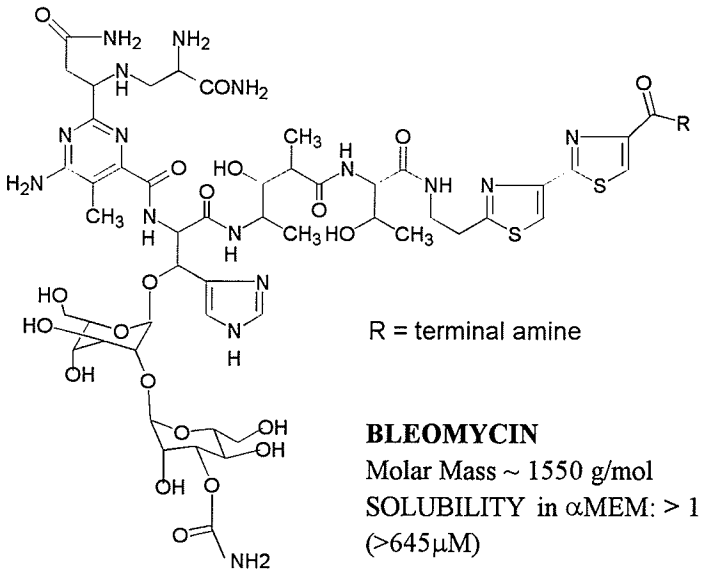
5-FLUOROURACIL
Molar Mass: 130.08 g/mol
SOLUBILITY in αMEM ~ 20 mM



VINBLASTINE
Molar Mass: 811.00 g/mol
SOLUBILITY in DMSO ~ 200 μM



HYDROXYUREA
Molar Mass: 76.06 g/mol
SOLUBILITY in αMEM: > 20 mM



R = terminal amine

BLEOMYCIN
Molar Mass ~ 1550 g/mol
SOLUBILITY in αMEM: > 1.0 mg/mL
(>645μM)

Figure 5.7.3.3.2 Illustration Of The Chemical Structure Of Drugs Incubated With CHO-AA8 And DZR3 For 48 Hours To Test For Cross-Resistance

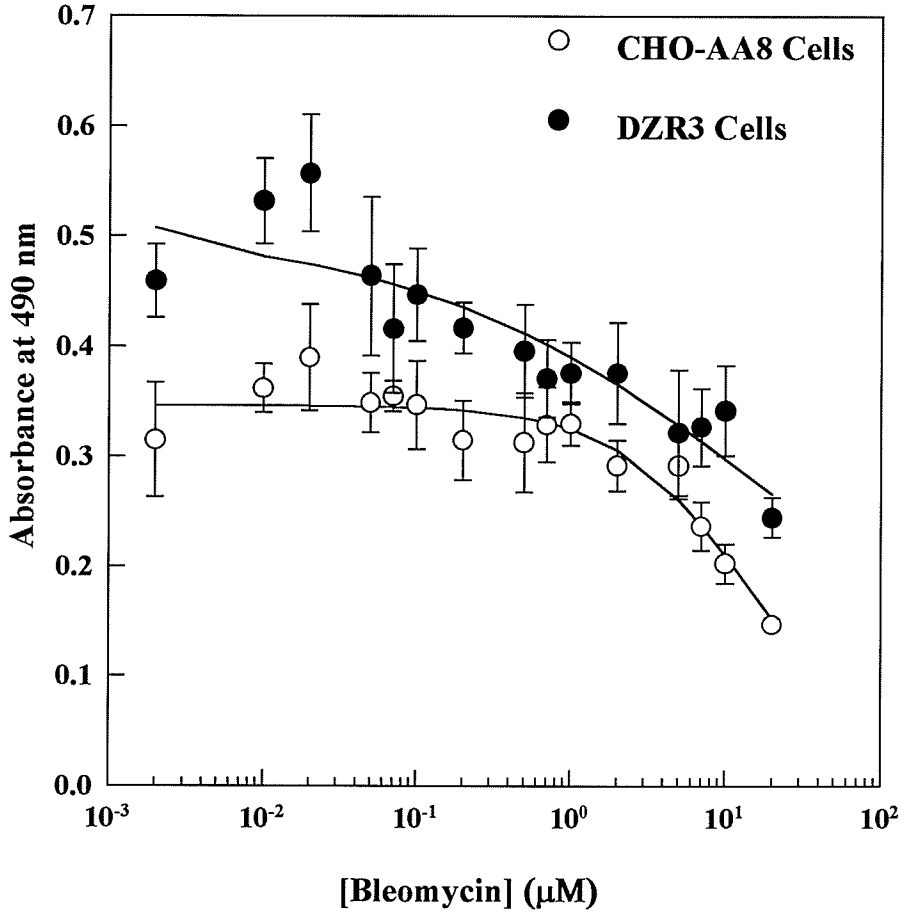


Figure 5.7.3.3.3 **MTT Assay Of Bleomycin-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 And DZR3 ICRF-187-Resistant Cells**

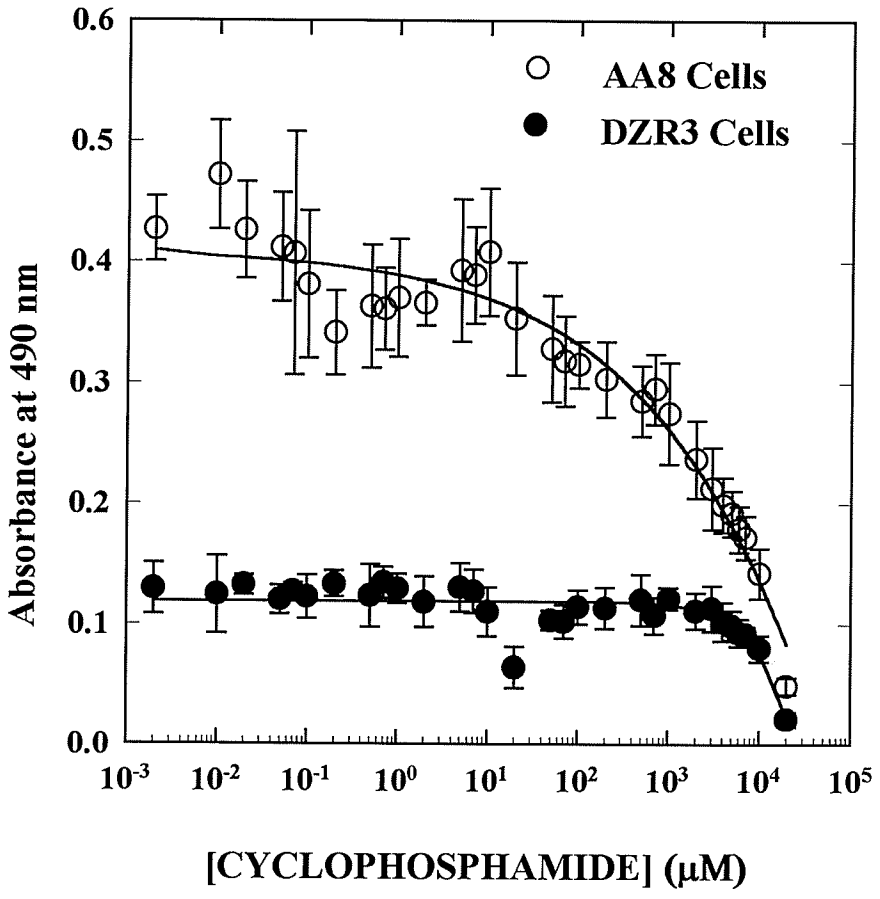


Figure 5.7.3.3.4 **MTT Assay Of Cyclophosphamide-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 And DZR3 ICRF-187-Resistant Cells**

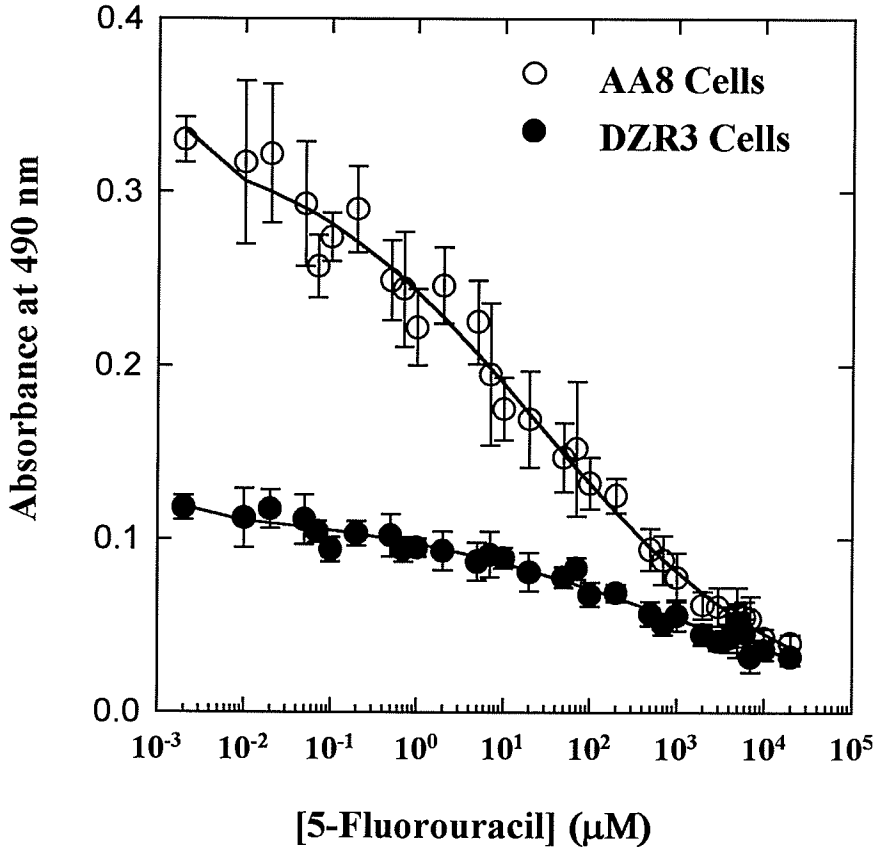


Figure 5.7.3.3.5 **MTT Assay Of 5-Fluorouracil-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 And DZR3 ICRF-187-Resistant Cells**

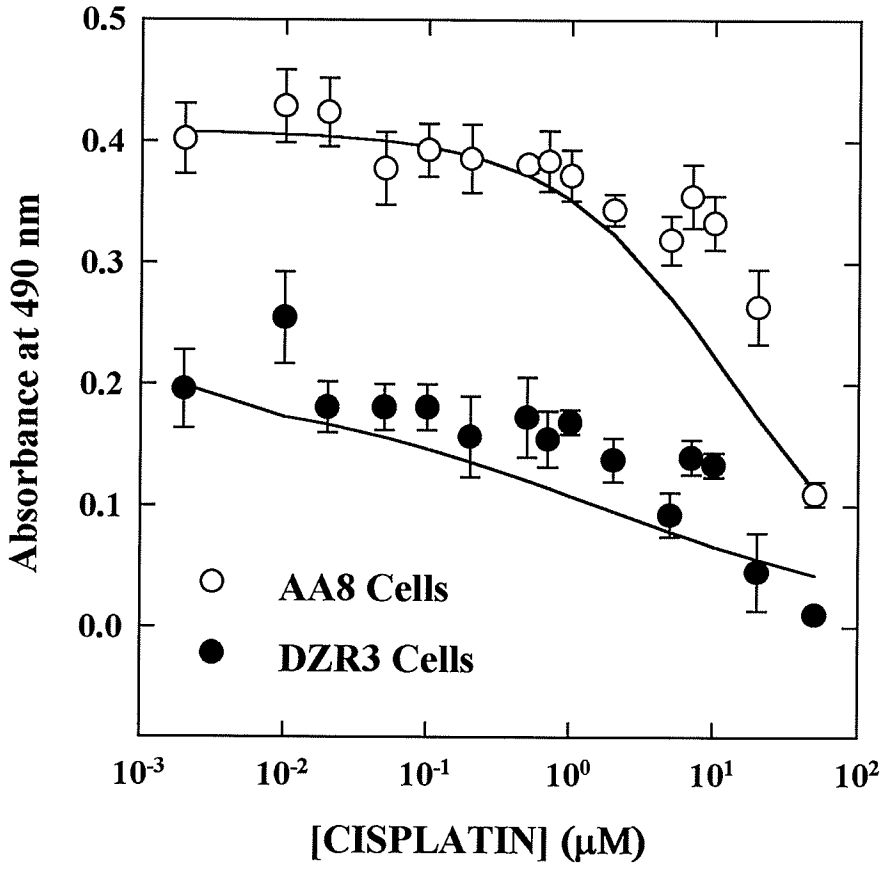


Figure 5.7.3.3.6 **MTT Assay Of Cisplatin-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 And DZR3 ICRF-187-Resistant Cells**

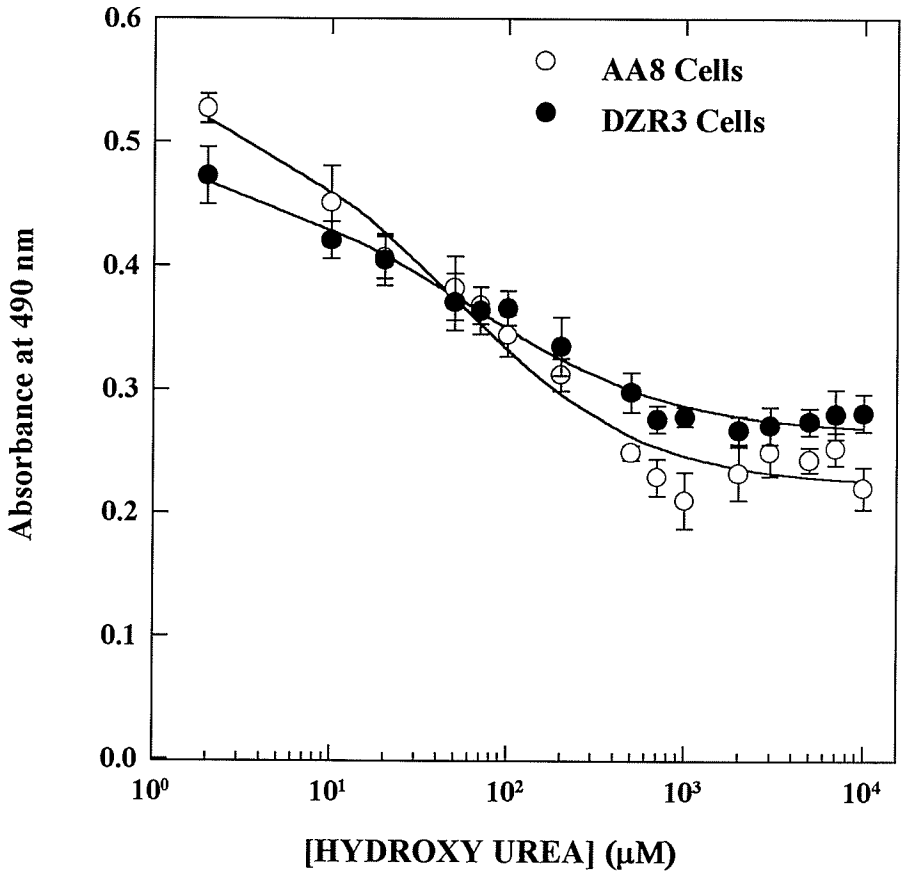


Figure 5.7.3.3.7 **MTT Assay Of Hydroxyurea-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 And DZR3 ICRF-187-Resistant Cells**

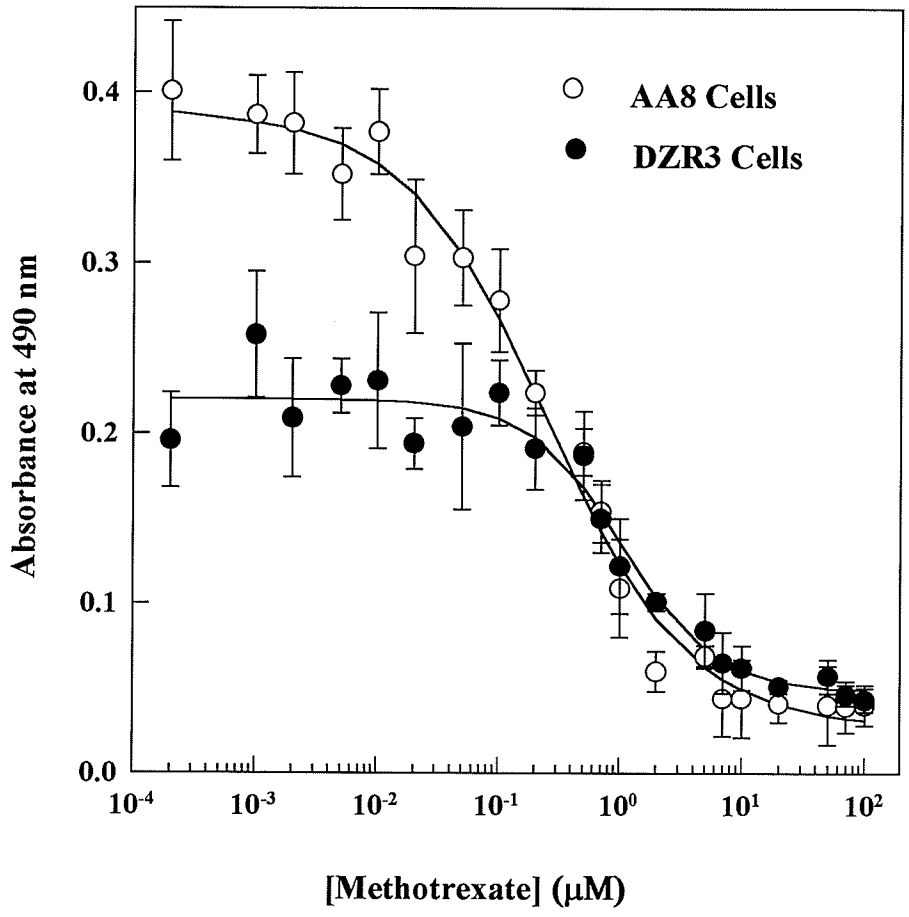


Figure 5.7.3.3.8 **MTT Assay Of Methotrexate-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 And DZR3 ICRF-187-Resistant Cells**

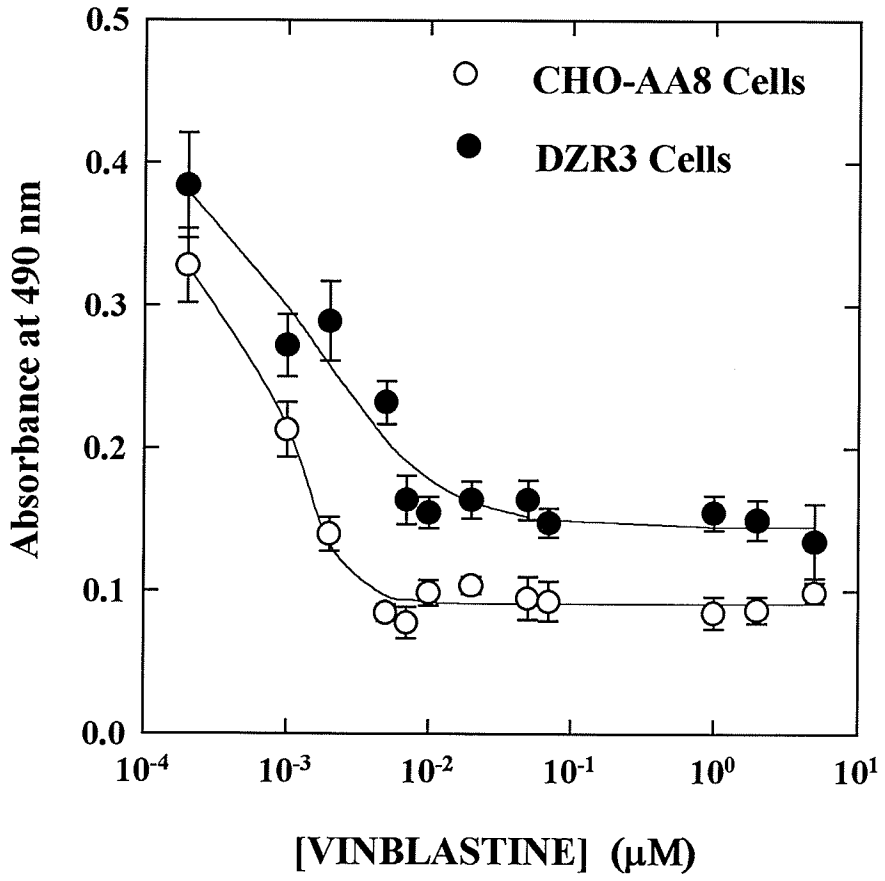


Figure 5.7.3.3.9 **MTT Assay Of Vinblastine-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 And DZR3 ICRF-187-Resistant Cells**

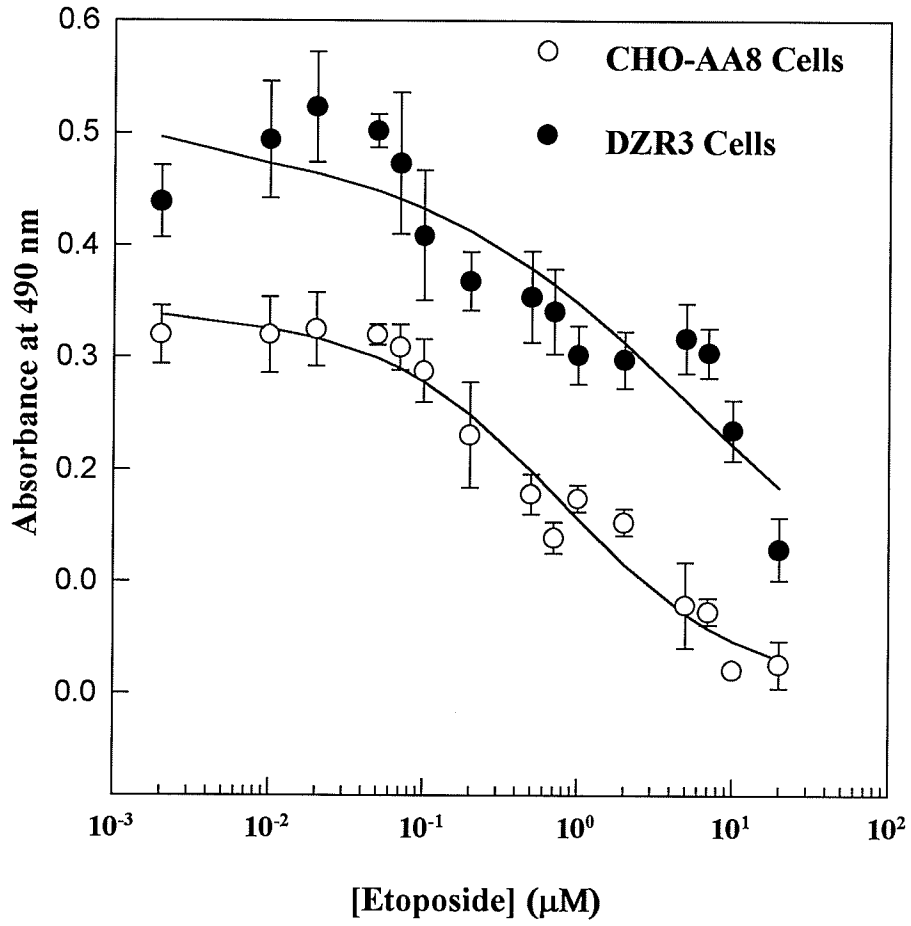


Figure 5.7.3.3.10 **MTT Assay Of Etoposide-mediated Cytotoxicity After 48 Hour Incubation With CHO-AA8 And DZR3 ICRF-187-Resistant Cells**

Table 5.7.3.3.1

50% Inhibitory Concentration Values And Exponential Factors For Drugs Incubated For 48 Hours With CHO-AA8 Cells

DRUG	IC_{50} Value (μM)	SEM	Exponential Factor	SEM
BLEOMYCIN*	0.99	0.01	0.35	0.01
CYCLOPHOSPHAMIDE	3250	740	0.37	0.01
5-FLUOROURACIL	25.4	11.0	0.30	0.03
CISPLATIN*	29.5	4.1	1.35	0.24
HYDROXYUREA	52.8	13.5	0.83	0.16
METHOTREXATE	0.25	0.04	0.74	0.09
VINBLASTINE	0.00104	0.00008	2.35	0.46
ETOPOSIDE	0.81	0.35	0.74	0.20
DOXORUBICIN	3.3	1.7	0.78	0.17
IDARUBICIN	0.1	0.03	0.64	0.12

* $d = Abs_{490min}$ was constrained to 0 to give a 3 parameter fit.

The IC_{50} values were computed using the equation,

$$ABS_{490obs} = (a-d)/(1+(x/c)^b)+d$$

where $a = Abs_{490max}$, $d = Abs_{490min}$, c = the IC_{50} value computed in the fit, b = the exponential factor, which gives the rate of increase of cytotoxicity as a function of the increase in drug dose, and x values are drug doses used in the experiment. These are selected from the column containing the drug concentrations in the Sigma plot program.

Table 5.7.3.3.2

50% Inhibitory Concentration Values And Exponential Factors For Drugs Incubated For 48 Hours With DZR3 Cells

DRUG	IC_{50} Value (μM)	SEM	Exponential Factor	SEM
BLEOMYCIN*	26.1	14.1	0.37	0.12
CYCLOPHOSPHAMIDE*	11,900	180	2.19	0.60
5-FLUOROURACIL*	520	160	0.26	0.03
CISPLATIN*	8.0	3.6	0.70	0.24
HYDROXYUREA	64.0	13.9	0.73	0.12
METHOTREXATE	1.0	0.1	0.76	0.12
VINBLASTINE	0.00184	0.00059	1.05	0.30
ETOPOSIDE*	6.36	3.0	0.74	0.20
DOXORUBICIN	3.3	1.6	0.78	0.12
IDARUBICIN	0.11	0.05	0.58	0.05

* $d = Abs_{490min}$ was constrained to 0 to give a 3 parameter fit.

A comparison of the differences in drug resistance of the between the CHO-AA8 cells and the DZR3 cells shows some differences in the IC_{50} values. Table 5.7.3.3.3 lists the resistance factors of the DZR3 cells over the CHO-AA8 cells in their ability to withstand the cytotoxic effects of these structurally unrelated drugs.

Table 5.7.3.3.3

A Comparison Of The Resistance Factors Of Several Unrelated Antitumor Drugs

Drug Tested	Resistance Factor
BLEOMYCIN	26
CYCLOPHOSPHAMIDE	3.7
5-FLUOROURACIL	21
CISPLATIN	0.27
HYDROXYUREA	1.2
METHOTREXATE	4.2
VINBLASTINE	1.8
ETOPOSIDE	7.9
DOXORUBICIN	13.2
IDARUBICIN	28.5

5.7.3.4 DISCUSSION

The ICRF-187-resistant cell line DZR3, though able to defend itself against several of the bisdioxopiperazines, is unable as a whole to protect itself in a comparable way against the insults of the majority of the common, but structurally unrelated antitumor drugs. A marginal increase in the resistance factors for some of these drugs shows a small amount of defensive capability, but a 26 fold resistance seen in bleomycin, a 13.2 fold resistance is seen against doxorubicin and a 28.5 fold resistance is seen against idarubicin. Bleomycin is believed to affect cells by intercalating the DNA and destroying

its three-dimensional integrity. This does not explain the 26 fold resistance. The majority of the resistance factors are significantly smaller than those observed in the bisdioxopiperazines tested. Hydroxyurea resistance is near unity, indicating no change in drug effects. This is not surprising, since hydroxyurea affects the ribinucleotide reductase functions. In the case of cisplatin, the DZR3 proved easier to kill than their wildtype counterpart, by a factor of approximately five times. The fact that any degree of resistance was developed at all is surprising since these agents are known not to affect topoisomerase II, which was the enzyme most likely to be affected by the selection process. The cells show a small degree of resistance to alkylating agents which is also unusual, since making cells resistant to an alkylating agent is difficult when it is done intentionally, much less inadvertently. The resistance of the cells to etoposide toxicity is somewhat less surprising since the drug affects topoisomerase II. Thus, any mutations within the resistant cells that afford a degree of protection against bisdioxopiperazines, which are believed to target topoisomerase II, might also extend protection against etoposide. The resistance against methotrexate is not expected since the drug affects the function of dihydrofolate reductase, and not topoisomerase II. Similarly, a small degree of resistance to vinblastine is unexpected since the drug targets the $\alpha\beta$ subunits of growing microtubules, and presumably has no direct relationship with resistance acquired against topoisomerase II inhibitors. Perhaps the resistance acquired to certain drugs unrelated to topoisomerase II is due to concomitant mutations which had occurred in other enzyme systems during the selection process. Though, these were not the intended affects,

the cells carrying these phenotypes may be better able to survive not only insult by bisdioxopiperazines, but other agents to a certain degree as well. Overall, these trends in resistance are in agreement with Creighton *et al.*, (1979) whose work showed that any increase in resisitance to other structurally unrelated compounds was small in comparison to that developed against bisdioxopiperazines in general.

5.8 TESTING FOR P-GLYCOPROTEIN CONTENT BY MEASURING ICRF-187 TOXICITY IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF VERAPAMIL

5.8 1 INTRODUCTION

A large number of multidrug resistant cells or tumors which acquire the MDR phenotype are shown to have an over expression of the *mdr1* gene product, P-glycoprotein. The P-glycoprotein (P-gp) is an energy dependent toxin pump found naturally in cells which process large amounts of toxins and metabolites, such as the liver, they kidney and cells in the gastrointestinal tract. This is beneficial to the cells in their disposal of natural toxins as well as xenobiotics, however the overexpression of this protein is problematic for those undergoing chemotherapy since it is these renegade cells which are able to rid themselves of the chemotherapy drugs. The unfortunate problem is compounded by the fact that the P-gp not only eliminates the agent responsible for its overexpression, but it is able to remove a whole host to antitumor agents, leaving a patient with little hope for surviving the illness.

The detection of the presence of P-gp is of value not only to the researcher seeking the solution to the MDR problem, but it is of critical clinical value since determination of the nature of the tumor prior to the initiation of chemotherapy may be the key to patient survival (Bosanquet, 1991). A tumor that can be examined by biopsy, tested for expression of P-gp before and after testing for tumor killing capacity of certain drugs, is able to give physicians a clear understanding of the tumor's capacity to defend itself. A battery of drugs shown to be lethal to the tumor grown *in vitro* can be used on the patient, eliminating guess work, patient discomfort as well as the acquisition of MDR phenotypes among the tumor cells.

One method used in the laboratory to detect multidrug resistance and clinically to enhance the effects of antitumor drugs on resistant cells is the calcium blocking agent, verapamil.The structure of verapamil is shown in Figure 5.8.3.2 This drug is able to block to afflux of toxins facilitated by P-gp, disabling the resistance mechanism acquired by the renegade cell. This antiarrythmic used to treat cardiac arrhythmia has shown value in research as well as in the treatment of patients undergoing chemotherapy.

In order to determine whether there is any P-gp activity in the DZR3 cells, an experiment was conducted measuring the cytotoxic effects of ICRF-187 on CHO-AA8 cells and compared with DZR3 cells. Cells were seeded and drugged with a broad range of ICRF-187. One set of plates received no verapamil, while the other two sets of plates received 3.0 and 10.0 μM doses of verapamil. If P-gp is present and functioning to protect the cell against ICRF-187 there should be a normal killing curve as expected for DZR3

incubated for 48 hours with ICRF-187, and there should be higher toxicity mediated by the ICRF-187 in the plates where 3.0 μM verapamil is used and higher toxicity still for the plates containing 10.0 μM verapamil. If there is no P-gp activity, all three cytotoxicity profiles should be the same.

5.8.2 METHODS AND MATERIALS

5.8.2.1 Seeding Of The Microtitre Plates With Cells

CHO-AA8 cells and DZR3 cells were grown to near confluence in a 25 cm^2 canted T-flask in αMEM containing 10% (v/v) calf serum. The medium was removed, the cells were washed with Dulbecco's PBS and then trypsinized. After quenching and centrifugation, the cells were resuspended in 10 mL of cell culture medium and counted six times. The average was taken and the cells were diluted appropriately to give a cell suspension of 20,000 cells mL^{-1}. Six microtitre plates were seeded with 100 μL volumes of each cell suspension to give well concentrations of 2,000 cells per well. Note that this was done for both sets of cells, with the total number of cells seeded requiring 12 microtitre plates. The cells were allowed to grow in darkness in the incubator overnight at 37°C in an atmosphere of 5% CO_2 and 95% (v/v)air.

5.8.2.2 Preparation Of The Drugs

After the 24 hour anchoring period, an 8 mL 20 mM stock solution of ICRF-187 was prepared in cell culture medium. The drug solution was diluted 10:1 serially in αMEM to 4 mL volumes of 2,000 μM, 200 μM, 20 μM, 2 μM and 0.2 μM

concentrations. A sample of verapamil was weighed out and dissolved by sonication in αMEM to a concentration of 100 μM, and filter sterilized by syringe filtration through a 0.2 μm filter. The verapamil solution was diluted to concentrations of 12 and 40 μM, which when delivered in 50 μL volume into a total of 200 μL, will give verapamil concentrations of 3.0 and 10.0 μM, respectively. Since 12 plates were prepared for the experiment approximately 150 mg of ICRF-187 crystal was weighed out.

5.8.2.3 Delivery Of the Drugs To The Microtitre Plates

Delivery of verapamil was made to two microtitre plates from substock solutions 12 and 40 μM solutions, whose final concentrations were 3.0 and 10.0 μM. The set of plates not receiving verapamil received 50 μL of αMEM. The ICRF-187 was delivered after the delivery of verapamil. ICRF-187 volumes added were no less than 10 μL and no greater than 50 μL. Any differences were made up to 50 μL with αMEM to give a well volume of 200 μL. The drug volumes delivered were to give a final range of concentration spanning from 0.01-20,000 μM plus a 0 μM control. Drugs were added using an 8 x 200 Gilson multichannel pipettor. The plates were allowed to incubate in the above mentioned conditions for 48 hours.

5.8.2.4 End-point Detection

After the 48 hour incubation of the cells with drug, 20 μL of 2.5 mg mL^{-1} MTT solution was added to the wells containing cells and allowed to incubate for another 4 hours. After this incubation, the medium was carefully aspirated off with an 18 gauge needle, without disturbing the cell layer, then 100 μL of spectral grade DMSO was added

to all the wells including those defined as blanks. After the formazan crystals were dissolved in the DMSO, the plates were read at λ= 490 nm minus λ = 650 nm.

5.8.3 RESULTS

The cytotoxicity plots for the CHO-AA8 and DZR3 cells incubated with 0 3.0 and 10.0 μM verapamil (whose structure is illustrated in Figure 5.8.3.1) and a range of ICRF-187 for 48 hours is shown in Figure 5.8.3.2. Table 5.8.3.1 shows the IC_{50} values and exponential factor computed for the curves. IC_{50} values for the CHO-AA8 cells were computed only between 0 and 50 μM since the rest was not relevant.

Table 5.8.3.1

IC_{50} Values And Exponential Factors For Cytotoxicity Experiments With CHO-AA8 and DZR3 Cells Drugged With 0, 3.0, Or 10.0 μM Verapamil, A Broad Range of ICRF-187 And Incubated For 48 Hours

Cell Type And Verapamil Dose	IC_{50} Value (μM)	SEM	Exponential Factor	SEM
CHO-AA8/ 0 μM Verapamil	1.88	0.19	2.1	0.3
CHO-AA8/ 3 μM Verapamil	1.49	0.20	2.1	0.5
CHO-AA8/ 10 μM Verapamil	1.42	0.01	1.7	0.3
DZR3/ 0 μM Verapamil	2800	200	3.0	0.6
DZR3/ 3 μM Verapamil*	2800	300	1.3	0.1
DZR3/ 10 μM Verapamil	2200	200	2.1	0.3

* $d = Abs_{490min}$ was constrained to 0 to give a 3 parameter fit.

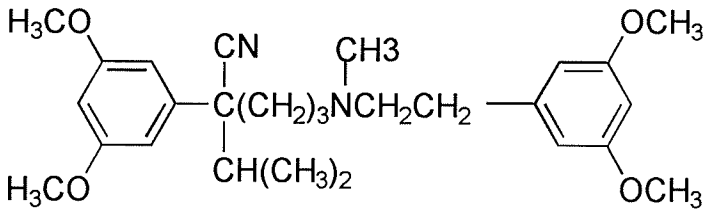
The IC_{50} values were computed using the equation,

$$Abs_{490obs} = (a-d)/(1+(x/c)^b)+d$$

where $a = Abs_{490min}$, $d = Abs_{490max}$, c = the IC_{50} value computed in the fit, b = the exponential factor, which give the rate of increase of cytotoxicity as a function of the increase in drug dose, and x values are experimental drug doses. These are selected from the column containing drug concentrations in the Sigma plot program.

Figure 5.8.3.1

Structure Of The Calcium Channel Blocking Agent Verapamil



VERAPAMIL
Molar Mass: 454.6 g/mol
Solubility in αMEM ~ 100 μM

As can be seen from the plot a well as from the IC_{50} values, there are small differences among the CHO-AA8 cell cytotoxicity plots. There is little difference between the 0 and 3.0 μM verapamil plates when considering the SEM however, there is a greater difference in the IC_{50} value between the 0 and 10 μM plots. The IC_{50} values differ by 0.5 μM, which is substantial considering the IC_{50} is only 1.9 μM at its highest.

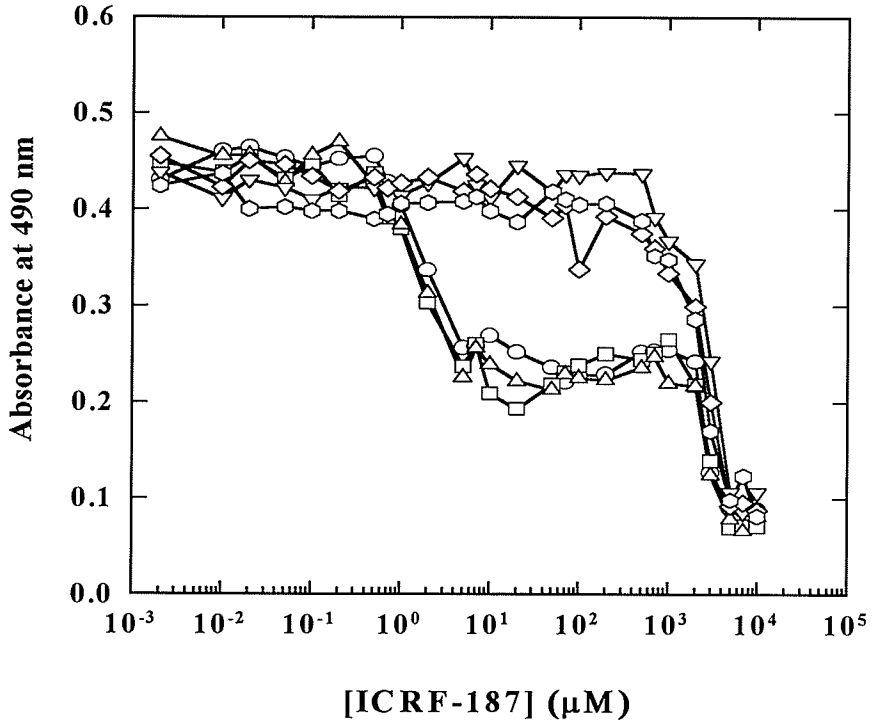


Figure 5.8.3.2 **MTT Assay For CHO-AA8 Cells And DZR3 Cells Incubated With 0, 3.0, And 10.0 μM Verapamil And A Range of ICRF-187 Over 48 Hours.**

In the case of the DZR3 plots the following trends are observed: The 0 and 3.0 μM doses both show IC_{50} values of 2,200 μM, while the 10 μM verapamil plate shows an IC_{50} of 2,800 μM. When considering the range of the SEM values, it in not reasonable to assume these differences in IC_{50} to be significant.

5.8.4 DISCUSSION

The experiments conducted with the drug-resistant cells shows that with enough verapamil, the cytotoxic capacity of ICRF-187 incubated with DZR3 cells can be increased. The difference in cytotoxicity between 0 and 10.0 μM is 21%. Obviously, the cytotoxicity of the drugs was not restored sufficiently to give IC_{50} values traditionally seen in the wildtype cells, but 21% is not insignificant. This might shed light on the partial differences observed in the degrees of resistance seen in CHO-AA8 cells and DZR3 cells with drugs that were discussed in 5.8.3. The drugs tested showed that there was a small degree of resistance exhibited by the resistant cells, not only to drugs associated with topoisomerase II inhibition, but to drugs believed to be the causative agents of P-gp overexpression. Again, the resistance was anywhere from less than 2 fold to as high as 26, but certainly not as high as in the case of most bisdioxopiperazines.

Perhaps there are two mechanisms by which the cell affords its drug resistance. What appears to be the primary mechanism is the mutation of topoisomerase II enzyme. This is reasonable since the selection was achieved by a drug which specifically targets topoisomerase II. This adequately explains the high resistance factors seen in DZR3

against the bisdioxopiperazines. The increase in resistance among drugs associated with P-gp could be explained by the small increase in toxicity seen upon the addition of verapamil to the ICRF-187 doses. Though, this is only a 21% difference, it may be enough when coupled with the primary line of defence, to sustain itself against not only against bisdioxopiperazines, but only to a small extent against bleomycin, cisplatin, methotrexate, etc. The resistance is small since as stated earlier, the drug used for selection is one associated with other mechanisms of defence and not P-gp. It might also contribute to the increase in IC_{50} values seen in the anthracyclines. Where the IC_{50} values for doxorubicin and idarubicin are 0.25 ± 0.08 μM and 0.0035 ± 0.0008 μM, respectively, the values seen for the DZR3 cells are 3.3 ± 1.7 μM and 0.10 ± 0.003 μM. For doxorubicin and idarubicin, these values represent resistance factors of 13.2 and 28.5, respectively. Perhaps, the resistant cell is able to pump out the toxic anthracyclines, as well as make changes to protect its topoisomerase II.

References

Abdella, B.R.J. and Fisher, J., *Environmental Health Perspectives* **64**: 3-18, 1985.

Alegria, A.E., Samuni, A., Mitchell, J.B., and Riesz, P., and Russo, A., *Biochemistry* **28**: 8653-8658, 1989.

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L.., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., and Boyd, M.R., *Cancer Research* **48**: 598-601, 1988.

Andrae, U., Singh, J., and Ziegler-Skylakakis., *Toxicology Letters* **28**: 93-98, 1985.

Arcamone, F. Cassinelli, G., and Franceschi, G., *International Symposium on Adriamycin,* pp. 9-22, Springer New York.

Barranco, S.C., *Pharmacology and Therapeutics* **24**: 303-319, 1984

Batist, G.,Tulpule, A., Sinha, B.K., Katki, A.G., Myers, C.E., and Cowan, K.H., *Journal of Biological Chemistry* **261**: 15544-15549, 1986.

Beck, W.T.,Cirtain, M.C., Look, A.T. and Ashmun, R.A., *Cancer Research* **39:** 2070-2076, 1979.

Beraldo, H., Garnier-Suillerot, A., Tosi, L., and Lavelle, F., *Biochemistry* **24**: 284-289, 1985.

Bezkorovainy, A. *Biochemistry of Nonheme Iron* pp. 1-120, Plenum Press, New York, 1980.

Biedler, J.L., Riehm H., Peterson, R.H.F., and Spengler, B.A., *Journal of the National Cancer Institute* **55**: 671-680, 1975.

References

Biedler, J.L., Meyers, M.B., and Spengler, B.A., in *Mechanisms of Drug Resistance in Neoplastic Cells*, pp.41-67, Academic Press, Inc. 1988.

Beijnen, J.H., Van der Nat, J.M., Labadie, R.P., and Underbery, W.J.M., *Anticancer Research*, **6**: 39-44, 1986.

Bosanquet, A.G, *The Lancet*, **337**: 711-714, 1991.

Brandi, G.,Luzzi, L., Gaicomoni, P., Albano, A., Cattabani, F., and Cantoni, O., *Mutation Research* **281**: 157-161, 1992.

Braughler, J.M. and Hall, E.D., *Free Radical Biology and Medicine* **6**: 289-301, 1989.

Brooks, S.C., Locke, E.R., Soule, H.D., *Journal of Biological Chemistry* **248**: 6251-6261, 1973.

Burke, T.G., Pritos, C.E., Sartorelli, A.C., and Tritton, T.R.,*Cancer Biochemistry and Biophysics* **9**: 245-255, 1987.

Buss, J.L. and Hasinoff, B.B., *Agents and Actions* **40**: 86-95, 1993.

Butler, M., *Mammalian Cell Biotechnology: A Practical Approach*, Oxford Press, 1981

Cantoni, O., Sestili, P., Cattabeni, F., Bellomo, G., Pou, S., Cohen, M., and Cerutti, P., *European Journal of Biochemistry* **182**: 209-212, 1989.

Carmichael, J.,*Cancer Research* **47**: 936-942, 1987.

Carter, S.B. unpublished.

Catalogue of Cell Lines & Hybridomas, 7th Edition, 1992.

Chance, B., *Physiology Review* **59**: 527-605, 1979.

References

Clarke, D.J., Johnson, R.T., and Downs, C.S. *et al., Journal of Cell Science* **105**: 563-569, 1993

Cole, S.P.C., Downs, H.F., Mirski, S.E.L., and Clements, D.J., *Molecular Pharmacology* **37**: 192-197, 1989.

Cole, S.P.C.,Chadna, E.R., Dicke, F.P., Gerlach, J.H., and Mirski, S.E.L.,*Cancer Research* **51**: 3345-3352, 1991.

Cowan, K.H., Batist, G., Tulpule, A., Sinha, B.K., Myers, C.E., *Proceedings of the National Academy of Science, U.S.A.* **83**: 9328-9332, 1976.

Creighton, A.M., Jeffery W.A., and Long, J., *Bisdioxopiperazines Medicinal Chemistry VI, Proceedings of the 6th International Symposium on Medicinal Chemistry*, Brighton U.K.,1979.

Cunningham, M.L. and Lokesh, B.R., *Mutation Research* **121**: 299-304, 1983.

D'Arpa, P. and Liu, L.F., *Biochimica et Biophysica Acta* **989**: 163-177, 1989.

Dalmark, M. and Storm, H.H., *Journal General Physiology*, **78**: 349-364, 1981.

Dawson, K.M., *Biochemical Pharmacology* **24:** 2244-2235, 1975.

Del Tacca, M., Danesi, R., Ducci., M., Bernardini, C., and Romani, A., *Pharmacology Research Communications* **17**: 1073-1085, 1985.

Demant, E.F.J., *European Journal Biochemistry* **142**: 571-575, 1984.

Demant, E.F.J. and Nørskov-Lauritsen, N., *FEBS Letters* **196**: 321-324, 1986.

Demant, E.F.J. *FEBS Letters* **176**: 97-100, 1984.

DeVonne, T.L. and Mouray, H., *Clin.Chem.Acta* **90**: 83-85, 1978.

References

Dietel, M., Siedel, A., and Nickelsen, M., *Proceedings of the American Association of Cancer Research.* **33**: 460, 1992.

Doroshow, J.H., *Cancer Research* **43**: 460-472, 1983.

Doroshow, J.H., *Proceedings of the National Academy of Science. U.S.A.* **83**: 4514-4518, 1986.

Dulhanty, A.M., Li, M., and Whitmore, G., *Cancer Research*, **49**: 117-122, 1989.

Dulik, D.M., Fenselau, C., and Hilton, J., *Biochemical Pharmacology* **35**: 3405-3409, 1987.

Dusre, L., Rajagopalan, S., Eliot, H.M., Covey, J.M., and Sinha, B.K., *Cancer Research* **50**: 648-652, 1990.

Eliot, H., Gianni, L., and Myers, C.E., *Biochemistry* **23**: 928-936, 1985.

Endicott, J.A. and Ling, V., *Annual Review of Biochemistry* **58**: 137-171, 1989.

Fantone, J.C. and Ward, P.A., *Oxygen-derived Radicals and Their Metabolites: Relationship to Tissue Injury.* Upjohn, Kalamazoo, 1985.

Fojo, A., Akiyama, S.I., Gottesman. M.M., Pastan. I.,*Cancer Research* **45**: 3002-3007, 1985.

Frei, E. III ., *Proceedings of the National Academy of Science U.S.A.* **82**: 2158, 1985.

Fridovich, I., *Annual Review of Biochemistry* **44**: 147-159, 1975.

Gabbay, E.J., Grier, D., Fingerle, R.E., Reimier, R., Levy, R., Pearse, S.W., and Wilson, W.D., *Biochemistry* **15**: 4209-4215, 1976.

References

Ghezzi, P., Donelli, M.G., Pantovotto, C, Faccinetii, T., and Garatinni, S., *Biochemical Pharmacology* **30**: 175-177, 1981.

Gianni, L., Corden B.J., and Myers, C.E.., *Reviews in Biochemical Toxicology*, **5**: 1-82, 1983.

Gianni, L., Zweier, J.L., Levy, A., and Myers, C.E., *Journal of Biological Chemistry* **260**: 6820-6826, 1985.

Gianni, L.,Vigano, L., Lanzi, C., Niggeler, M., and Malatesta, V., *Journal of the National Cancer Institute* **80**: 1104-1111, 1988.

Gille, J.J.P., Berkel, C.G.M., Mullaart, E., Vijg, J., and Joenje, H., *Mutation Research*, **214**: 89-96, 1989.

Gorbsky, G.J., *Cancer Research* **54**: 1042-1048, 1994.

Greenwald, R.A., *Free Radical Biology and Medicine* **8**: 201-209, 1990.

Halliwell, B. and Gutteridge, J.M.C., *Biochemical Journal* **219**: 1-14, 1984

Halliwell, B.and Gutteridge, J.M.C., *Free Radicals in Biology and Medicine*, Clarendon, Oxford, 1985.

Halliwell, B. and Gutteridge, J.M.C., *Archives Biochemistry and Biophysics* **246**: 614-636, 1986.

Hasinoff, B.B. and Davey, J.P., *Biochemical Journal* **250**: 827-834, 1988.

Hasinoff, B.B. and Davey, J.P, *Biochemical Pharmacology* **37**: 3663-3669, 1988.

Hasinoff, B.B., *Agents and Actions.* **26**: 378-385, 1989.

Hasinoff, B.B., *Biochemistry and Cell Biology* **68**: 1331-1336, 1990.

References

Hasinoff, B.B., *Agents and Actions* **29**: 374-381, 1990.

Hasinoff, B.B., Reinders, F.X., and Clarke, V., *Drug Metabolism and Disposition* **19**: 74-80, 1991.

Hasinoff, B.B., *Drug Metabolism and Disposition* **21**: 883-888, 1993.

Hasinoff, B.B., *Journal of Pharmaceutical Sciences* **83**: 64-67, 1994.

Hasinoff, B.B.and Kala, S.V., *Agents and Actions* **39**: 72-81, 1993.

Hassan, H.M. and Fridovich, I., *Enzymatic Basis of Detoxication*, **Vol. I**, Academic Press, New York, 1980.

Hayflick, L. and Moorhead, P.S., *Experimental Cell Research* ***25***: 585, 1961.

Heck, M.M.Hittleman, W.N., and Earnshaw, W.C., *Journal Cellular Biology* **103**: 2569-2581, 1986.

Heck, M.M.,and Earnshaw, W.C., *Proceedings of the National Academy of Science U.S.A.*, **85**: 1086-1090, 1988.

Hellman, K. and Field, E.O., *Journal of the National Cancer Institute* **44**: 539-543, 1970.

Herman, E.H., El-Hage, A.N., Creighton, A.M., Witiak, D.T., Ferranse, V.J., *Research. Communications:Chemical Patholology and Pharmacol*ogy **48**: 39-55, 1985.

Hill, B.T., Shellard, S.A., Hosking, L.K., Dempke, W.C.M., Fichtinger-Schepman, A.M.J., Tone, T., Scanlon, K.J., and Whelan, R.D.H., *Cancer Research* **52**: 3110-3118, 1992.

Huang, Z.-X., May, P.M., Quinlan, K.M., Willians D.R., and Creighton, A.M., *Agents and Actions* **12**: 536-542, 1982.

References

Hunt, R.C. and Marshall-Carlson, L., *Journal of Biological Chemistry* **261**: 3681-3686, 1986.

Ishida, R., Miki, Tetsuo, Narita, T., Yui, R.., Sato, M., Utsumi, K.R., Tanabe, K., *Cancer Research* **51**: 4909-4916, 1991.

Ishimi, Y., Ishida, R., and Toshiwo, A., *Molecular and Cellular Biology* **12**: 4007-4114, 1992.

Ito, A., Watanabe, HG., Naito, M., and Niato, Y., *Gann* **72**: 174-175, 1981.

Juliano, R.L. and Ling, V., *Biochimica et Biophysica Acta* **455**:152-162, 1976.

Kalyanaraman, B., Sealy, R.C., and Sinha, B.K., *Biochimica et Biophysica Acta* **799**: 270-275, 1984.

Kartner, N., Evernden-Porelle, D., Bradley, G., and Ling, V., *Nature* (Lond.) **316**: 820-823, 1985.

Keizer, G.H., Pinedo, H.M., Schuurhuis. G.J., and Joenje, H., *Pharmacology and Therapeutics* **47**: 219-231, 1990.

Krishan, A.and Frei.E. III, *Cancer Research* **35**: 497-501, 1975.

Le Bot, M.A.,Bégué, J.M., Kernaleguen, D., Roberts, J., Ratanasavanh, D., Airiau, J., Riche, C., and Guillouzo, A., *Biochemical. Pharmacology* **37**: 3877-3887, 1988.

Ling, V. and Thompson, L.H., *Journal Cellular Physiology* **83**: 103-116, 1974.

Lown, J.W. *Advanced Free Radical Biology and Medicine* **1**: 225-264, 1985.

Lum, B.L. Gosland, M.P., Kaubisch, S., and Sikic, B.I., *Pharmacotherapy* **13 (2)**: 88-109, 1993.

References

Marks, D.C., Belov, L., Davey, M.W., Davey, R.A., and Kidman, A.D., *Leukemia Research* **16:** 1165-1173, 1992.

Martins, E.A.L., Chubatsu, L.S., and Menegheni, R., *Mutation Research*, **250**: 95-101, 1991.

Martinsson, T., Dahllof, B., Wettergren, Y., Leffler, H., and Levan, G., *Experimental Cell Research* **158**: 382-394, 1985.

May, P.M., Williams, K.G.,and Willims D.R., *Inorganica Chimica Acta* **46**: 221-228, 1980.

Mimnaugh, E.G., Gram, T.E., and Trush, M.A., *Journal of Pharmacology and Experimental Therapeutics* **226**: 806-816, 1983

Mirski, S.E.L.,Gerlach, J.H., and Cole, S.P.C.,*Cancer Research* **47**: 2594-2598, 1987.

Moody, C.S. and Hassan, H.M., *Proceedings of the National Academy Science U.S.A.* **79**: 2855-2859, 1982.

Morley, C.G.D.and Bezkorovainy, A., *International Journal of Biochemistry* **17**: 553-564, 1985.

Mosman, T., *Journal of Immunological Methods* **65**: 55-63, 1983.

Muindi, J.R.F., Sinha, B.K., Gianni, L., and Myers, C.E., *FEBS Letters* **172**: 226-230, 1984.

Murray, S.L., DuVall, E.M., and Slater, L.M., *Cancer Chemotherary and Pharmacology* **13**: 60-70, 1984.

References

Myers, *C.E.*, Gianni, L., Simone, C.B., Klecker, R., and Greene, R., *Biochemistry* **21**: 1707-17-13, 1982.

Nakagawa, K.,Saijo, N., Tsuchida, S., Sakai, M., Tsunokawa, Y.,Yokota, J., Muramatsu, M., Sato, M.,Terada,M., and Tew K.D., *Journal of Biological Chemistry* **265**: 4296-4301, 1990.

Nakayama, T., Niimi, T., Osawa, T., and Kawasishi, S., *Mutation Research*, **281**: 77-80, 1992.

Nogrady, T., *Medicinal Chemistry A Biochemical Approach*, 2nd Ed. Oxford University Press, 1988.

O'Brien, S., Kantarjian, H., Freireich, E., Johnston, D. Nguyen, K., and Beran, M., *Cancer Research* **52**: 4130-4134, 1991.

Ohno, R., Yamada, K., Harano, M., Shirakawa, S., Tanaka, M., Oguri, T., Kodera, Y., Mitomo, Y., Ikeda, Y., Yokamaku, S., Kamiya, O., Kobayashi, M., Saito, H., Kimura, K., *Journal of the National Cancer Institute* **84**: 435-438, 1992.

Okamoto, K. and Ogura, R., *Journal of Nutritional Science and Vitaminology* **31**: 129-137, 1985

Pearse, A.G.E. in *Histochemistry, Theoretical and Applied*, 3rd Ed., Chapter 20, Edinbugh: Churchill Livingston, 1972.

Potmesil, M., Kirschenbaum, S., Israel, M., Levin, M., Khetarpal, V.K., and Silber, R., *Cancer Research* **43**: 3528-3533, 1983.

Rowe, T.C., Kupfer, G., and Ross, W., *Biochemical Pharmacology* **33**: 3137-3142, 1984.

References

Rubinstein, L.V., Shoemaker, R.H., Paull, K.D., Simon, R.M., Tosini, S., Skehan, P., Scudiero, D.A., Monks, A., and Boyd, M.R., *Journal of the National Cancer Institute* **82**: 1113-1118, 1990.

Salonen, J.T., Nyyssönen, K., Korpela, H.,Tuomilehto, J., Seppänen, R., and Salonen, R., *Circulation* **86**: 803-811, 1992.

Sahlin, M., Gräslund, A., Ehrenberg, A., and Sjöberg, B.M., *Journal of Biological Chemistry* **257**: 366-369, 1982.

Sapino, A., Peitribiasi, F., Bussolati, G., and Marchiso, P.C., *Cancer Research* **46**: 2526-2531, 1986.

Sato, S., Iwaizumi, M., Handra, K., and Tamura, Y., *Gann* **68**: 603-608, 1977.

Saburi, Y., Nakagawa, M., Ono, M., Muramatsu, M., Kohno, K., and Kuwano, M.., *Cancer Research* **49**: 7020-7025, 1989.

Scudiero, D.A., Shoemaker, R.H., Paull, K.D., Monks, A., Tierney, S., Hofziger, T.H., Currens, M.J., Seniff, D., and Boyd, M.R..,*Cancer Research* **48**: 4827-4833, 1988.

Seglin, P.O., *Methods in Cell Biology*, **Vol XIII**, Ed. Prescott, D.M. Academic Press, New York, 1976.

Sehested, M., Jensen, B.B., Sørensen, B.S., Holm, B., Friche, E., and Demant, E.J.F., *Biochemical Pharmacology* **46**: 389-393, 1993.

Shea, T.C., Kelley, S.L., and Henner,W.D., *Cancer Research* **48**: 527-533, 1988.

Sinha, B.K., *Chemical-Biological Interactions* **30**: 66-77, 1980.

Sinha, B.K. and Gregory, J.L., *Biochemical Pharmacology* **30**: 2626-2636, 1985.

References

Sinha, B.K., Trush, M.A., Kennedy, K.A., and Mimnaugh, E.G., *Cancer Research* **44**: 2892-2896, 1984.

Sinha, B.K and Sik, R.H., *Biochemical Pharmacology* **29**: 1867-1868. 1980.

Sinha, B.K., Katki,A.G., Batist, G., Cowan, K.H.,and Myers, C.E., *Biochemistry* **26**: 3776-3781, 1987.

Slater, T.F., Sawyer, B., and Straulli, U., *Biochemia et Biophysica Acta* **77**: 383-343, 1976.

Smith, E., Stratford, I. J., and Adams G.E., *British Journal of Cancer* **42**: 568-573, 1980.

Speyer, J.L.Green, M.D., Kramer, E., Rey, M., Sanger, C., Ward, C., Dubin, N., Ferrans, V., Sanger, C., Stecy, P., Zeleniuk-Jacquotte, A., Wernz, J., Fiet, F., Slater, W.,Blum, S., *New England Journal of Medicine* **319**: 745-752, 1988.

Speyer, J.L., Green, M.D., Zeleniuk-Jacquotte, A.,Wernz, J.,Rey, M.,Sanger, C., Sanger, C., Kramer, E., Ferrans, V., Hochster, H., Meyers, M., Blum, R.H., Feit, F., Attubatto, W., Burrows, W., Muggia, F.M., *Journal of Clinical Oncology* **10**: 117-127, 1992.

Sugioka, K. and Nakano, M., *Biochimica et Biophysica Acta*, **713**: 333-343, 1982.

Taft, S.A., Ling, S.Y., and Griffiths. D.T., *Mutation Research, DNA Repair* **255**:257-264, 1991.

Tanabe, K., Ikegami, Y., Ishida, R, and Toshiwo, A., *Cancer Research* **51**: 4903-4908, 1991.

Tannock, I. *Cancer Research*, **42**: 4921-4926, 1982.

References

Tewey, K.M., Rowe, T.C., Yang. L., Halligan, B.D., and Liu, L.F., *Science* **226**: 466-468, 1984.

Timson, J., *Mutation Research* **32**: 115-132, 1975.

Tsuruo, T., Iida, M., Nujiri, M., Tsukagushi, S., and Sakurai, Y., *Cancer Research* **43**: 2905-2910, 1983.

Twentyman, P.R., Fox, N.E., Wright, K.A., and Bleehen, N.M., *British Journal of Cancer* **53**: 527-537, 1986.

van der Kraaij, A., Mostert, L.J., van Eijk, H.G., Koster, J.F., *Circulation* **78**: 442-449, 1988.

Van Renswoude, J. Bridges, K.R., Harford, J.B., Klausner, R.D., *Proceedings of the National Academy of Science U.S.A.* **79**: 6168-6190, 1982

Vistica, D.T., Skehan, P., Scudiero, D.A., Monks, A., Pittman, A., and Boyd, M.R., *Cancer Research* **51**: 2515-2520, 1991.

Wadler, S., Green, M.D., and Muggia, F.M., *Cancer Research*, **46**: 1176-1181, 1986.

Wadler, S., Green, M.D., Basch, R., and Muggia, F.M. *Biochemical Pharmacology* **36:** 1495-1501, 1987.

White, K. and Creighton, A.M., *British Journal of Cancer* **34**: 232, 1976

Wilson, W.R., Thompson, L.H., Anderson, R.F.,nad Denny, W.A., *Journal of Medicinal Chemistry* **32**: 31-38, 1989.

Woessner, R.D., Mattern, M.R., Mirabelli, C.K. Johnson,, R.K., and Drake, F.H.,*Cell Growth & Differentiation.* **2**: 209-214, 1991.

Yalowich, J., unpublished

Yamashiro, D.J.,Tychko, B., Fluss, S.R., and Maxfield, F.R., *Cell* **37**: 789-800, 1984.

Yang, L-Y and Trujillo, J.M., *Cancer Research* **50**: 3218-3225, 1990.

Young, R.C., Ozols, R.F., and Myers, C.E., *New England Journal of Medicine* **305**: 139-153, 1981.

Zweier, J.L., Gianni, L., Muindi, J., and Myers, C.E., *Biochimica et Biophysica Acta*, **884**: 326-336, 1986.

Zwelling, L.A., Michaels, S., Erickson, L.C., ngerleider, R.S., Nichols, M., and Kohn, K.W., *Biochemistry, **20**:* 6553-6563, 1981.