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**CHARACTERIZATION OF HUMAN OVARIAN CANCER BY
MAGNETIC RESONANCE SPECTROSCOPY USING *IN*
VITRO AND *EX VIVO* MODELS**

by

JULIA C. WALLACE, B.Sc., M.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment
of the requirements for the degree of

Doctor of Philosophy

Department of Physics
Carleton University
Ottawa-Carleton Institute of Physics
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submitted by

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in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

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ABSTRACT

This thesis examines the potential role of Magnetic Resonance Spectroscopy (MRS) in diagnosing ovarian cancer and detecting cellular changes that occur following the induction of resistance in cells.

Proton (^1H) MRS characteristics of four human ovarian cancer cell lines were measured. Two of these cell lines were from untreated ovarian cancer patients and the remaining two cell lines were cisplatin (a common anti-cancer drug) resistant mutants developed from the two parental cell lines. The drug resistant cells exhibited different metabolite profiles compared to their sensitive parental cell lines. In particular, changes in the levels of glutathione, taurine, glutamate and lipids were observed. These changes were consistent with known cellular mechanisms of resistance.

An additional level of complexity in modelling *in vivo* ovarian cancer was attained by measuring the MR spectra of cells in different growth phases and multicellular spheroids of increasing diameter. Cells in exponential growth phase exhibited significantly different spectra from those in plateau phase. Spectra from small spheroids closely resembled the spectra from exponentially growing cells. Larger spheroids with necrotic centres had elevated lipid spectral intensities.

^1H MR spectra of *ex vivo* normal post-menopausal ovaries and ovarian neoplasms were obtained to provide biochemical information which could be used to discriminate between normal and cancerous tissue. Using linear discriminant analysis (LDA), classification of normal and cancer was accomplished with a sensitivity of 100% and a specificity of 95% and overall accuracy of 98%. In addition, LDA distinguished untreated ovarian cancer from recurrent ovarian cancer with sensitivity 92%, specificity 100% and

overall accuracy 97%. With removal of the single "fuzzy" sample, accuracy was increased to 100%. Thus ^1H MRS shows promise in characterizing the properties of chemotherapy resistant ovarian cancer cells.

With the long term goal of assessing the optimal treatment protocol for resistant cells, a perfusion system was designed and constructed which could maintain viable growing cells for over 72 hours. Cells were embedded in calcium alginate beads and the biological characteristics of these embedded cells were assessed. ^{31}P spectra of drug-sensitive and -resistant ovarian cancer cells were measured to monitor energy and membrane metabolites. The resistant mutants showed significantly elevated levels of phosphocreatine (PCr) and phosphodiesterases (PDE) ($P < 0.05$). ^{31}P spectra were also measured on perchloric extracts of the same cell lines and, in agreement with the results obtained on perfused cells, elevated levels of PCr and PDE were observed.

This study indicates that MRS can be used to distinguish between classes of ovarian cancer (*ex vivo*) and can detect differences between drug -sensitive and -resistant cell lines (*in vitro*). Applications of this knowledge to *in vivo* measurements could lead to non-invasive diagnosis and monitoring of ovarian cancer.

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GLOSSARY

adriamycin	an anti-cancer drug
ascites	accumulation of serous fluid in the abdominal cavity
benign	property of a neoplasm that it does not invade adjacent structures or spread to distant sites
biopsy	small piece of tissue used to establish diagnosis
carcinoma	malignant neoplasm which arises from epithelial cells
chemical shift	variation in the Larmor frequency of a particular isotope depending on the chemical environment it is in
cisplatin	an anti-cancer drug
culture	propagation of cells in defined media
EMT6/Ro	mouse tumour cell line
<i>ex vivo</i>	removed from the living body
HEPES	<i>N</i>-(2-hydroxyethyl)piperazine-<i>N'</i>-2-ethanesulfonic acid
histology	study of tissues, microscopic anatomy
<i>in vitro</i>	observable in a test tube, removed from the natural environment
<i>in vivo</i>	within the living body
invasive	property of malignant neoplasms by which cells begin growing in the connective tissue stroma of the tissue of origin
malignant	ability of a neoplasm to invade adjacent tissues and spread to distant sites
metastases	a subpopulation of malignant cells which has detached itself from the primary tumour
necrosis	death and decomposition of a portion of a tissue
neoplasm	abnormal mass of cells which exhibit uncontrolled growth
primary neoplasms	neoplasm which originated from tissue at given site
sensitivity	true positive fraction or the fraction of disease which is discovered
specificity	true negative fraction or the fraction of healthy patients identified

LIST OF ABBREVIATIONS

δ	chemical shift
μ_i	individual magnetic moments
1D	one-dimensional
^1H	proton
2D	two-dimensional
^{31}P	phosphorus
ATP	adenosine triphosphate
BSO	buthionine sulfoximine
D_2O	deuterium oxide
DMEM	dubecco's modified eagles medium
emf	electromotive force
FBS	fetal bovine serum
FID	free induction decay
GPC	glycerophosphorylcholine
GPE	glycerophosphorylethanolamine
H	external field
H_0	dc magnetic field
H_{rf}	alternating radio frequency field
IC_{50}	Drug dose required to kill 50% of cells
ID_{50}	Radiation dose required to kill 50% of cells
J	coupling constant
K	1024 (referring to computer memory)
LDA	linear discriminant analysis
LOO	leave-one-out method
LQ	linear quadratic model
M	macroscopic magnetization vector
MRS	magnetic resonance spectroscopy
NAD	nicotinamide adenine dinucleotide
NBS	newborn calf serum

NRC	National Research Council
NTP	nucleoside triphosphate
ORCC	Ottawa Regional Cancer Centre
PABA	p-aminobenzoic acid (chemical shift reference)
PBS	phosphate buffered saline
PCA	perchloric acid
PCho	phosphorylcholine
PCr	phosphocreatine
PDE	phosphodiester
PE	plating efficiency
PEth	phosphorylethanolamine
Pi	inorganic phosphate
PME	phosphomonoester
ppm	parts per million
rf	radio frequency
SF	survival fraction
SNR	signal-to-noise ratio
T₁	longitudinal or spin-lattice relaxation time
T₂	transverse or spin-spin relaxation time
T_d	population doubling time
W	words (referring to computer memory)

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CHAPTER 1 INTRODUCTION

1.0 Introduction

Two of the key problems facing clinicians treating ovarian carcinoma are late detection of the disease and resistance of most tumours to current treatments. This thesis examines the potential role of Magnetic Resonance Spectroscopy (MRS) in diagnosing ovarian cancer and detecting cellular changes that occur following the induction of resistance in cells.

Ovarian cancer is the sixth leading cause of cancer deaths among women in Canada (1). The lifetime probability of a Canadian woman developing ovarian cancer is 1.6%. While in the early stages it is asymptomatic, and at diagnosis 95% of ovarian cancers measure more than 5 cm in diameter (2) and over 75% have metastasized beyond the pelvis. Treatment for advanced stage ovarian cancer is usually surgery followed by platinum based combination chemotherapy (typically with cisplatin or cisplatin analogues) or, less frequently, by radiation. Despite advances in treatment, the survival rate has not improved significantly in the past twenty years. The five year survival rate for women with regional disease is 41% which is reduced to 21% for disease with distant metastases (3).

Resistance to drugs and radiation is a major cause of treatment failure. Approximately one third of patients with advanced ovarian cancer have tumours which are intrinsically resistant to treatment (4). The remaining two thirds of patients initially respond to treatment, but the majority will suffer a relapse. Fewer than 10% of the recurring tumours will respond to second-line chemotherapy due to the development of resistance. These cells become resistant either specifically to the inducing drug or they can, in addition,

become broadly cross resistant to a range of structurally unrelated drugs and radiation. Thus in the majority of patients there is a subpopulation of ovarian cancer cells that is either intrinsically resistant or can acquire resistance during treatment

A non-invasive method of distinguishing ovarian cancer cells from normal ovarian epithelial cells would prove useful for screening high risk patients. If, in addition, the presence of resistant cells could be detected, this would be an important step towards predicting the outcome of treatment. Proton MRS (^1H -MRS) has been used successfully for monitoring changes in cellular lipids and metabolites of transformed cells (5,6) and for diagnosing lung (7), colorectal (8), brain (9,10), thyroid (11) and cervical cancer biopsy samples (12,13). In this study, MRS methods will be used to identify ovarian cancer and to search for spectral "fingerprints" indicative of cellular resistance.

For a "bench-to-bedside" approach, three models of ovarian cancer were chosen: a) human ovarian cancer cells grown on tissue culture dishes, b) human ovarian cancer cells grown as multicellular spheroids and c) human ovarian cancer biopsy samples removed during surgery. Cell models provide information on ovarian cancer without introducing complications due to differences in vasculature, immune response and host tissue-tumour interactions. Cells grown on tissue culture dishes and as multicellular spheroids are maintained in a well controlled environment. The supply of nutrients, oxygen as well as the pH level can be duplicated for each preparation of the same or different cell lines. Spheroids are aggregates of cells grown at tissue-like densities and provide additional levels of complexity as well as three-dimensional cell to cell contact which is present in tumours. Cells on the periphery of spheroids are well supplied with nutrients and oxygen and are able to proliferate. Closer to the centre of the spheroids,

cells may not be supplied with adequate nutrients to support active division. Larger spheroids may contain a necrotic core. Thus there are inhomogeneities in the microenvironments of the spheroids. Fortunately, as a result of the spherical shape of the spheroids, they have radial symmetry and can be easily modelled. Like cells grown on plates, the growing conditions of spheroids can be well controlled. Biopsy samples provide a further level of complexity. Ovarian cancer samples removed during surgery are extremely heterogeneous. They contain many cell types in addition to cancer cells, including immune cells and fat cells, and thus more closely represent the biochemical picture *in vivo*. Before outlining the layout of this thesis some details on the diagnosis and treatment of ovarian cancer will be presented.

1.1 Classification of Ovarian Cancer

Ovarian cancers are classified according to histological type, stage and grade. Histological type describes the cell from which an ovarian tumour was derived. Neoplasms arise from four main compartments of the ovary: the surface epithelium, germ cells, sex cord-stroma and non-specific mesenchyme (see Figure 1.1). The majority (85-90%) of malignant ovarian cancers are epithelial in origin (14). They can be further divided into histological subtypes as seen in Table 1.1 (14). The histological type is not a good indicator of a patient's prognosis.

Prognostic factors which are important include the stage, grade and extent of residual disease following surgery. The stage is a measure of the amount of spread of the disease and is assigned during surgery. The five year survival rates of patients decreases markedly with increasing stage. Surgical stages and their corresponding survival rates can be found in Table 1.2.

Figure 1.1 Representation of an ovary showing the major compartments from which neoplasms arise.

Table 1.1 Histogenic classification of ovarian neoplasms (14)

<u>Surface Epithelium</u> Serous Tumour Mucinous Tumour Endometrioid Tumour Clear Cell Tumour Brenner (or transitional) Cell	<u>Germ Cell</u> Dysgermina Embryonal CA (rare in ovary) Teratoma (mature or immature) Endodermal Sinus Tumour Choriocarcinoma	<u>Sex Cord Stroma</u> Granulosis Cell Tumour Thecoma Fibroma Sertoli-Leydig Tumour
<u>Unclassified</u> Small Cell Carcinoma	<u>Non-Specific Mesenchyme</u> Hemangioma Leiomyoma Lymphoma	

Table 1.2 Stages and survival rates of ovarian cancer (15,16)

Stage	Characteristic	Five year Survival (%)
I	Growth Limited to Ovaries	90
IA	Growth limited to one ovary; no ascites; no tumour on external surfaces, capsule intact	
IB	Growth limited to both ovaries; no ascites; no tumour on external surfaces, capsule intact	
IC	Tumour either stage IA or IB but with tumour on the surface of one or both ovaries, or with capsule ruptured, or with ascites containing malignant cells or with positive peritoneal washings	
II	Growth involving one or both ovaries with pelvic extension	70
IIA	Extension or metastases to the uterus or tubes	
IIB	Extension to other pelvic tissues	
IIC	Tumour either stage IIA or IIB but with tumour on the surface of one or both ovaries, or with capsule ruptured, or with ascites containing malignant cells or with positive peritoneal washings	
III	Tumour involving one or both ovaries with peritoneal implants outside the pelvis or positive retroperitoneal or inguinal nodes; superficial liver metastases; tumour is limited to the true pelvis but with histologically verified malignant extension to small bowel or omentum	25
IIIA	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces	
IIIB	Tumour of one or both ovaries; histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes negative	
IIIC	Abdominal implants bigger than 2 cm in diameter or positive retroperitoneal or inguinal nodes	
IV	Growth involving one or both ovaries with distant metastases	10

Histological grade describes the percentage of undifferentiated cells in a section of tumour (17). The prognosis for poorly differentiated tumours (grades III and IV) is worse than for well differentiated tumours (grade I). An additional factor which determines patient outcome is the extent of residual disease following surgery and this should be less than 2 cm in diameter for treatment to be successful (18). With the exception of histological type, each of these factors supplies independent prognostic information and can be used to evaluate the risk of recurrence.

Most ovarian neoplasms are benign. Diagnosis of benign versus malignant tumours is usually made on the basis of gross and microscopic examination

A group of epithelial ovarian tumours with features between those of benign and frankly malignant ovarian neoplasms represents 15% of ovarian cancers (14). These cancers, also called proliferative cystadenomas or borderline tumours, have low malignant potential.

1.2 Treatment

For patients with advanced stage disease, the first line of treatment is usually surgery followed by platinum-based combination chemotherapy or, less frequently, abdominopelvic radiotherapy. The rationale for both methods of treatment is to kill tumour cells while sparing the damage to normal tissue. As a first approximation, tumour cell killing follows first order kinetics, i.e. for a given dose of radiation or drugs, a constant fraction of the cells will be destroyed (19). In Chapter 3 a more detailed model of cell killing will be discussed.

With the exception of bone marrow cells, cells in the gastrointestinal tract and hair follicles, tumour cells proliferate much more rapidly than normal tissue cells. Cell cycle specific treatments, which are effective against rapidly growing tumours, take advantage of this to kill tumour cells preferentially. Radiation, for example, kills relatively more cells in certain cell cycle phases. Radiation damage to cells manifests itself as lesions in the DNA. These lesions include single and double strand breaks which are considered to be the most toxic. Lethal damage is typically expressed during cell division. Sensitivity to DNA damage varies throughout the cell cycle (19-21), and these variations are exploited with treatments involving multimodalities (hyperthermia, drugs and radiation) and fractionated radiotherapy (22,23).

Slowly growing tumours have a high proportion of nonproliferating cells and are relatively insensitive to cell cycle specific treatments. Hence combination therapy is usually chosen which includes both cycle specific and non-cycle specific drugs.

Currently, the most effective anticancer drug used to fight ovarian cancer is cisplatin, a non-cycle specific drug (see Figure 1.2a). Cisplatin is a neutral square-planar compound (24). The precise mechanism by which cisplatin achieves cell killing is not known conclusively. DNA is generally believed to be the target (25). Following a hydration step, intracellular cisplatin covalently binds to proteins, glutathione (a thiol important for radioprotection and drug detoxification) and DNA. Binding to DNA leads to the formation of inter- and intra-strand links, usually between guanines, which may or may not be lethal (see Figure 1.2b.).

The single most important reason for treatment failure is resistance to the chosen treatments. As the primary method of treating ovarian cancer involves

a

b

Figure 1.2 Structure of the anti-cancer drug cisplatin or *cis*-dichlorodiammineplatinum: a) the molecule and its hydrated form (15) b) mechanisms of cisplatin binding to DNA (26).

cisplatin or its analogues, resistance to this drug will be discussed in more detail.

1.3 Drug Resistance

It is estimated that 90% of cancer deaths can be attributed to resistance (27). Drug-resistant tumours can be separated into two classes, those with 'intrinsic' resistance (or a subpopulation of cells within the tumour which are resistant prior to treatment) and those that exhibit 'acquired' resistance. Both 'intrinsic' and 'acquired' resistance are clinically apparent in ovarian cancer (27). Cells can be resistant to one class of drugs or can be resistant not only to the inducing drug but also a broad spectrum of structurally unrelated drugs and cross resistant to radiation.

Clinically, drug resistance is between two and ten fold (i.e. cells require up to ten times the dose of drug to achieve the same level of killing). The administration of higher doses is not possible because of potentially lethal side effects (28).

1.4 Mechanisms of Resistance: Possible Roles for MRS

Tumour drug resistance may result from changes in drug metabolism in the patient and spread of tumour cells to inaccessible sites in addition to cellular changes (see Table 1.3) (27). Cellular mechanisms include decreased drug accumulation, increased DNA repair and increased drug detoxification (4). These mechanisms are outlined in the following chart along with the possible changes that may be detectable using MRS.

Table 1.3 Mechanisms of resistance to chemotherapy (27).

Host factors**Altered pharmacokinetics****Decreased drug absorption-activation****Enhanced drug excretion-degradation****Altered binding-transport proteins****Host tumour factors****Metastases of cells to "sanctuary sites"****Cellular factors****Decreased drug accumulation****Decreased drug influx****Increased drug efflux****Altered cellular metabolism****Decreased activation****Increased degradation****Cytoplasmic-nuclear activation****Glutathione****Metallothioneins****Proteins****DNA repair****Tolerance to DNA damage****Altered cellular target**

Hence ^1H MRS may detect different levels of glutathione and phospholipid metabolites in resistant cells and phosphorus (^{31}P) MRS may be used to monitor the changing energy requirements needed to transport drugs from cells or repair DNA damage.

Some clinical and experimental applications of ^1H and ^{31}P MRS are described in the following sections. Other nuclei of interest to cancer research (^{13}C , ^{23}Na , ^{195}Pt , ^{19}F) will not be reviewed here.

1.5 Magnetic Resonance Studies of Human Tumours

Magnetic resonance spectroscopy can measure metabolic characteristics of tissues noninvasively. There have been over 700 MR studies (29) of human cancers. Many of these studies have examined whether human cancers have

metabolic characteristics that are either specific to cancer or can be used to predict or monitor treatment response.

^1H MRS provides information on total creatine, total choline, lipids, glutamate, glutamine and many other cell components. ^{31}P MRS can be used to monitor the energy status of cells (phosphocreatine (PCr), inorganic phosphate (Pi), nucleoside triphosphate (NTP)), phospholipid metabolites (phosphomonoesters (PME), phosphodiester (PDE)) and intracellular pH.

1.5.1 Proton MR Studies

Brain tumours have been studied extensively by ^1H MRS. Primary brain tumours are characterized by low *N*-acetyl aspartate, high total choline and low total creatine compared to contralateral normal brain tissue (see 29 for review). One-dimensional (1D) and two-dimensional Correlated Spectroscopy (2D COSY) ^1H spectra have been used successfully to classify brain tumour biopsies according to pathological diagnosis. Highly malignant tumours were discriminated from less malignant tumours on the basis of lipid cross peaks in the 2D spectra and elevated ratios of lipid/choline in the 1D spectra (9). However, a recent study of high grade astrocytomas has shown that increased lipids may be associated with the development of necrotic areas. (10,30). This suggests that classification of tumours by MRS may not only reflect the histopathological diagnosis but also some other biochemical processes.

Fewer tumours outside the brain have been investigated because of large lipid signals which may mask interesting spectral information. This problem can be overcome (albeit invasively) by examining tissue extracts (with the lipid components removed) which have sharp and easily quantified resonances. For example, an MR study of lung cancer extracts distinguished between different

types of lung cancer and normal lung using ratios of choline, creatine, alanine and glycine (7). Caution must be taken when extrapolating data from extracted tissue to the *in vivo* situation because MR visibilities of the extracted metabolites may be different from those of intact cells and, during extraction, some hydrolysis of acid or base sensitive components may occur. Work is actively being done on tissues that have not undergone chemical extraction including discriminating between cervical (12,13), ovarian (31), thyroid (11) and colon (32-34) biopsy samples with different pathologies.

In a study of human colorectal carcinoma and adenoma cell lines, it was found that differences in MR visible lipid, metabolites and cell surface fucosylation correlated with tumorigenicity and reduced differentiation (6)

In a combined ^1H - ^{31}P MR study of cell extracts from drug-sensitive and -resistant breast cancer cells the latter had elevated levels of PCr, Creatine (Cr), adenosine triphosphate (ATP) and adenosine diphosphate (ADP) and reduced concentrations of phosphorylcholine (PCho) and phosphorylethanolamine (PEth) (35).

1.5.2 Phosphorus MR Studies

Cancers typically have high PME and PDE levels which may be related to the high turnover rate of cancer cells or changes in phospholipid membrane composition (29). They also have reduced levels of PCr (29). High pH measurements and long T_2 values were an excellent predictor of eventual response to therapy. Most frequently, the early metabolic change that preceded response was the decrease in PME (29,36,37). In Chapter 6, the ^{31}P MR spectra of drug -sensitive and -resistant ovarian cancer cells will be examined and ^{31}P MR studies of particular relevance will be discussed

1.6 Hypotheses, Aims and Outline

This study examines the hypothesis that ^1H MR spectra can be used to distinguish between normal ovarian tissue and ovarian cancer and, further, that classification can be improved using multivariate techniques. This study will be an important step towards evaluating the clinical role of *in vivo* ^1H MRS for ovarian cancer.

Secondly, this thesis investigates the hypothesis that changes leading to 'acquired' resistance may result in differences in drug detoxifying systems and membrane-related components which can be observed by ^1H MRS. In addition, new energy requirements and differences in energy metabolites can be monitored by ^{31}P MRS. A successful demonstration of different MRS characteristics between drug -sensitive and -resistant cells may provide a basis for predicting and/or monitoring drug response *in vivo*.

Hence the specific aims of this study are to

- characterize 1D and 2D COSY ^1H MR spectra of human ovarian cancer cell lines grown *in vitro*
- compare MR spectra from drug -sensitive and -resistant ovarian cancer cells
- examine differences in ^1H MR spectra resulting from tumour heterogeneity and necrosis using biopsies and multicellular spheroids
- classify ovarian cancer and normal ovary biopsies removed during surgery using multivariate analyses of the MR spectra
- determine whether MRS can distinguish between untreated and recurrent ovarian cancer biopsies
- design and implement a cell perfusion system for measuring ^{31}P MR spectra of growing cells

- measure differences in energy requirements and phospholipid metabolites between treatment-sensitive and -resistant ovarian cancer cells using ^{31}P MRS.

This thesis is organized into the following format, with the underlying aim of starting with the simplest model of ovarian cancer and progressing through to a more complicated system which more closely reflects ovarian cancer *in situ*.

MR and multivariate theory will briefly be presented in Chapter 2. In Chapter 3, the ^1H MR spectra of two human ovarian cancer cell lines are compared to spectra from their resistant mutants. These cells were grown to confluency in monolayers.

In Chapter 4 the influence of growth phase on ^1H MR spectra of ovarian cancer cells grown in monolayers or as multicellular spheroids is examined.

^1H MR spectra of ovarian cancer biopsies are studied in Chapter 5. These samples are classified using multivariate techniques to improve classification.

Chapter 6 returns to the *in vitro* cell model. In this chapter cells were kept viable for 72 hours in a perfusion system and ^{31}P MR spectra of a sensitive and resistant pair of human ovarian cancer cell lines are presented.

Finally in Chapter 7, conclusions and future directions are presented.

CHAPTER 2   THEORY

2.0   Introduction

The theory section is divided into two parts. The first discusses some aspects of MR theory with particular emphasis on details relevant to biological systems. The second part briefly introduces multivariate theory and some applications to cancer research.

2.1   MR Theory

2.1.0   Introduction

For a sample in thermal equilibrium in a static magnetic field, $H_0 = H_0 \hat{k}$, there is a net magnetization parallel to the field. Individual nuclear magnetic moments (μ_i), that sum to give the net magnetization, precess about the z-axis (see Figure 2.1a) at a frequency that is characteristic for the nucleus. This frequency is known as the Larmor frequency. To study a particular nucleus, (in this thesis ^1H and ^{31}P , both spin $1/2$), a radiofrequency (rf) field is applied perpendicular to the static field at the precession frequency of the nucleus under investigation. This pulse of rf is configured to tip the net magnetization into the x-y plane. If the sample is in a coil oriented perpendicular to the static field, the precessing magnetization induces an electromotive force (emf) in the coil (Figure 2.1b). The signal obtained is called the free induction decay or FID (Figure 2.1c). The Fourier transform of this FID gives the spectrum which is of primary interest in this thesis.

Figure 2.1 MR spectroscopy: measuring a free induction decay a) In the presence of a static magnetic field the magnetic moments precess about the direction of the field, b) Following the application of a 90° rf pulse, the precessing magnetization in the transverse plane induces a signal in the coil, c) The free induction decay (FID) generated by the precessing magnetization

Magnetic resonance spectroscopy will be described below classically. More rigorous treatments (which involve quantum mechanics) can be found (37-39).

Certain nuclei possess an angular momentum and a corresponding magnetic moment. The behaviour of an ensemble of these nuclei in an external magnetic field can be described by the phenomenological equations of Bloch (40)

$$\frac{d\mathbf{M}}{dt} = \gamma \mathbf{M} \times \mathbf{H} - \frac{M_x \mathbf{i} + M_y \mathbf{j}}{T_2} - \frac{M_z - M_0}{T_1} \mathbf{k} \quad 2.1$$

$\mathbf{M}$ = ensemble nuclear magnetization

M_0 = equilibrium magnetization

M_x, M_y, M_z = components of $\mathbf{M}$ along $\mathbf{i}, \mathbf{j}$ and $\mathbf{k}$ axes

$\mathbf{H}$ = magnetic field

γ = gyromagnetic ratio

T_1, T_2 = characteristic relaxation times
defined below

The first term in Equation 2.1 describes the torque experienced by the ensemble magnetization in the presence of the external field. The external field, $\mathbf{H}$, consists of a static field, $\mathbf{H}_0$, and an alternating rf field, $\mathbf{H}_{rf}$, applied perpendicular to the static field, which can be considered as a field rotating in the x, y , plane at a frequency ω_{rf} . Solutions to 2.1 are usually found by transforming to a coordinate system rotating at the frequency of the applied field. This coordinate system is denoted $x', y', z'=z$. When the resonance condition is met (i.e. when the frequency of the rf is equal to the Larmor frequency of the nucleus under study), the z component of the field in the rotating frame is zero. The phase of the rf can be chosen so that the field is applied along the rotating x' -axis ($H_{rf} \mathbf{i}'$ in the rotating frame). An rf pulse along

the x'-axis will produce a rotation of the net magnetization in the y'-z plane (see Figure 2.2). A pulse of duration τ_p will produce a rotation angle of

$$\theta = \gamma H_x' \tau_p \quad 2.2$$

A pulse resulting in a rotation of 90° is referred to as a 90° or $\pi/2$ pulse and a pulse with a corresponding rotation of 180° is known as a 180° or π pulse. These pulse names will be used when discussing specific pulse sequences used in this study.

The second term in Equation 2.1 represents the decay of the transverse magnetization (the x and y components of magnetization), i.e. the loss of phase coherence, resulting from interactions of the spins with each other. Energy is exchanged within the spin system. The characteristic time for this decay is called the transverse relaxation time, T_2 . The dominant mechanism for T_2 relaxation of ^1H and ^{31}P nuclei in biological molecules is the dipole-dipole interaction (41). Molecular motions cause fluctuations of the dipoles and therefore modulate T_2 .

The third term in Equation 2.1 describes the trend of the magnetization towards its equilibrium value M_0 and is characterized by the longitudinal relaxation time T_1 . This process involves an exchange of energy between the spins and their environment (also known as the lattice).

Concepts of linear response (40) and Fourier transform theory can be applied to MRS. The FID induced in the receiver coil can be considered the impulse response of the system and the complex spectrum is the frequency response function (see Figure 2.3). These functions form the Fourier transform pair given in Equation 2.3

Figure 2.2 Pulse (90°) of rf applied along the x' -axis tipping the net magnetization in the y' - z plane (adapted from 43)

Figure 2.3 Relation between frequency and response of a linear system. The real and imaginary components of the complex frequency spectrum are Hilbert transformations of each other (denoted $\mathcal{H}$). The impulse response (FID) is a cosine or sine Fourier transformation of the frequency response (denoted $\mathcal{F}_c$ or $\mathcal{F}_s$) (adapted from reference 40).

$$\begin{aligned}
 S(\omega) &= \int_{-\infty}^{\infty} s(t) e^{-i\omega t} dt \\
 s(t) &= \frac{1}{2\pi} \int_{-\infty}^{\infty} S(\omega) e^{i\omega t} d\omega
 \end{aligned}
 \tag{2.3}$$

where $S(\omega)$ is the frequency spectrum, $s(t)$ is the FID and ω is the angular frequency as shown in Figure 2.3.

Hence the FID of a sample following a 90° pulse can be Fourier transformed to give the MR spectrum of the sample. For an idealized FID that decays monoexponentially, i.e. $s(t) = s(0)\exp(-t/T_2)$, the resulting line shape in the frequency domain is a single Lorentzian line.

$$s(\omega) = \frac{T_2}{[1 + T_2^2(\omega - \omega_0)^2]}
 \tag{2.4}$$

where ω_0 is the frequency of the peak maximum, i.e. the resonant frequency. Thus, the line width at half height is equal to $2/T_2$. Molecules in solution often have Lorentzian lineshapes resulting from the motional averaging of the dipole-dipole interactions, whereas immobilized molecules give rise to broader signals. Thus, spectral widths provide some information on molecular mobility. This will be discussed more fully later.

To enhance signal resolution or signal-to-noise ratios in the spectra the data can be filtered. This is most easily accomplished by multiplying the time domain signal by an appropriate time domain filter function: exponential line broadening for improved signal to noise or the Lorentzian -Gaussian Transformation for resolution enhancement (44).

2.1.1 Interpretation of the MR Spectrum

Many factors influence the MR spectrum, resulting in changes in frequency, area and shape of an MR signal (42). The origin of these factors, be they the chemical environment or the mobility of the species under study, is often the information that is of interest. Only three effects will be discussed; chemical shift, spin-spin coupling and line shape. Other factors which affect the observed spectrum include magnetic field strength, field and sample heterogeneities and the presence of material with different magnetic susceptibility or paramagnetic impurities (38, 45).

Chemical Shift, δ

A complex MR spectrum, such as that seen in Figure 2.4, arises because nuclei in molecules are partially shielded from the external field by electrons within the molecule. Electron orbitals or chemical ring currents close to a given nucleus result in a shift of the effective field experienced. Thus, these nuclei require different magnetic fields to achieve resonance than does a bare nucleus. The difference in magnetic field required is proportional to the strength of the applied field.

The chemical shift is a measure of the nuclear shielding divided by the applied field and is given with respect to a reference compound

$$\delta = \frac{(B_{\text{sample}} - B_{\text{ref}})}{B_{\text{ref}}} \times 10^6 \text{ ppm} \quad 2.5$$

where B denotes the magnetic induction field.

Figure 2.4 ^1H MR spectrum of 2008 ovarian cancer cells (5×10^7) measured at 37° on a wide bore Bruker AM360. Assignments of metabolites made from literature values, addition of standard compounds and from the 2D COSY measurements.

The predominant contribution to proton chemical shifts comes from diamagnetic shielding. Protons have a small range of chemical shifts (~ 10 ppm) and are present in a large number of molecular environments in cells. Thus, proton spectra are extremely complex. A phosphorus spectrum, on the other hand, is comparatively simple, and has a fairly wide range of chemical shifts (30 ppm).

In the simple ^1H MR spectrum shown in Figure 2.5, the groups of resonances at different resonant frequencies are assigned to different functional groups of ethyl benzene (46). Chemical shift dispersion and MR detection sensitivity increase with the applied field. Human subjects are usually investigated at field strength between 0.1 and 1.5 Tesla whereas the studies for this work were performed at much higher fields.

The electronic environment surrounding a compound will alter if the degree of protonation changes (46). Thus, chemical shifts of some resonances are pH sensitive. Of particular importance in biological ^{31}P MRS is the pH sensitivity of inorganic phosphate, the chemical shift of which is frequently used as a measure of inter- and intracellular pH.

Spin-spin couplings

Spin-spin or scalar couplings lead to the fine structure or splittings of resonance peaks in the MR spectra (47). Two nuclei in different environments (i.e. having different chemical shifts) may have coupled magnetic moments, causing perturbation to the precession frequency, and line splitting. The frequency separation of the splitting induced by coupled nuclei is called the spin-spin coupling constant J which is in frequency units, Hz. The

Figure 2.5 ^1H MR spectrum of ethyl benzene showing the resonances from the aromatic, methylene and methyl groups. This spectrum illustrates chemical shift, δ , and spin-spin coupling, J (adapted from 46).

number and amplitude of the split lines depends on the spins of the coupling nuclei and the number of coupled nuclei. This interaction is independent of the size and direction of the external magnetic field.

First order spectra arise from coupled nuclei whose resonances have a frequency difference much greater than the magnitude of the coupling constant. This condition is not fulfilled for the second order and consequently more complex spectra.

Fine structure can be observed on the resonances from the aromatic, methylene and methyl groups of ethyl benzene in Figure 2.5 (46). Interaction with the two CH_2 protons, which have 3 spin states when in a magnetic field, splits the resonance from the CH_3 protons into three with relative intensities 1:2:1. The complex splitting of the aromatic resonance is an example of a second order spectrum.

Resonance lines in the ^{31}P spectra from cell extracts (Chapter 6) clearly show splittings, as do the resonance peaks from the reference compound used for the cell and biopsy studies (Chapters 3-5). Splittings of other resonances are masked by line broadening. Spin-spin couplings are also of fundamental importance in 2D correlated spectroscopy, which will be discussed in Section 2.1.2.

Line Shapes : Example of a Proton Spectrum of Cells

Within a motionally restricted system, neighbouring magnetic moments produce a local field at the site of a given nucleus. The strength of the local field depends on the magnitudes and orientations of neighbouring magnetic moments and their positions relative to the given nucleus. The range in local fields experienced by the ensemble gives rise to dipolar-broadened lines. In

solid powders, these lines tend to be Gaussian in shape. Lipids in membranes exhibit non isotropic planar symmetry leading to the observed broad "superLorentzian" line shapes (45).

Whole cells reorient in water too slowly to produce motional averaging of the dipole-dipole interactions (45). Thus, the spectral lineshapes from components within cells depend on the motions of the components themselves. A spectrum of rat adenocarcinoma R13762 cells is shown in Figure 2.6. The high resolution spectrum is thought to arise from rapid isotropic tumbling of cellular components. On the MR timescale, rapid motions lead to the spins experiencing an average local field due to neighbouring spins. Under these conditions, Lorentzian line shapes are observed. By contrast, membrane lipids and integral membrane proteins are motionally restricted and contribute to the broad underlying spectrum. In biological samples, both broad and narrow resonances can be present (45).

In addition to reduced molecular mobility, broadening of the resonance peaks may result from overlapping peaks in the MR spectrum, making interpretation more difficult. Two-dimensional MR spectroscopic methods can aid in identifying metabolite resonances that are poorly resolved in the 1D spectra (48). However, long measurement times are required to obtain adequate signal-to-noise and spectral resolution in 2D spectra.

Figure 2.6 ^1H MR spectrum of rat mammary adenocarcinoma 13762 cells a) complete spectrum, b) expanded plot of central region (reference 45)

2.1.2 Two-Dimensional MR Spectroscopy

Energy Levels in an AB system

For a spin $\frac{1}{2}$ nucleus there are two possible orientations in a magnetic field which are given by the magnetic quantum number m_I : $m_I = +\frac{1}{2}$ (which is the lower and more stable state and is denoted by the symbol α) and $m_I = -\frac{1}{2}$ which is denoted β . An energy level diagram for a pair of spin $\frac{1}{2}$ nuclei, A and B, is shown in Figure 2.7. There are four states given by the four orientations of the nuclei ($\alpha\alpha$, $\alpha\beta$, $\beta\alpha$, $\beta\beta$) and six possible transitions between the states. These are the single quantum transitions (A_1 , A_2 , B_1 , B_2), the zero quantum transition ($\alpha\beta$ to $\beta\alpha$) and the double quantum transition (between $\beta\beta$ and $\alpha\alpha$).

Using the vector model from before and the classical description, if a 90° pulse is applied to a system in thermal equilibrium the magnetization vector will precess in the x-y plane or in other words the x and y components precess coherently. In terms of the energy level diagram, there is phase coherence between the α and β states of one of the nuclei (single quantum coherence) and therefore an NMR signal is induced (50).

Coherence can be transferred to zero, double and even higher order transitions with the use of multiple pulses. For example, suppose a 90° pulse creates phase coherence across A_2 (i.e. between $\alpha\alpha$ and $\beta\beta$). Application of a second pulse will transfer the single quantum coherence to all possible transitions. Thus, with two pulses, phase can be transferred from $\alpha\alpha$ to $\beta\beta$ ($\Delta M = 2$) or $\alpha\beta$ to $\beta\alpha$ ($\Delta M = 0$).

Figure 2.7 Energy level diagram for two interacting nuclei (49)

2D MR Spectroscopy

The pulse sequence for measuring the 2D correlated spectra will be discussed subsequently but in short is simply two 90° pulses separated by a varying time interval. The 2D spectrum can be thought of as a 'map' of coherence transfer during the application of the second pulse (40).

The 2D technique used in this work is called Homonuclear Correlated Spectroscopy (COSY). The basis of this technique is that magnetization /coherence is transferred between coupled spins.

The COSY experiment uses the following pulse sequence

This sequence can be separated into four stages (44): preparation, evolution, mixing and detection. During the preparation stage the spins align with the static field prior to the initial pulse. During the evolution period, the spin system is allowed to evolve freely. The length of the evolution period, t_1 , is increased incrementally. The mixing process consists of the second 90° pulse which transforms single, zero or multiple quantum coherences into observable transverse magnetization (40), i.e. single quantum coherences from one nucleus can be transferred to all possible transitions including the observable single quantum coherences of coupled nuclei. During the detection period t_2 , the FID for each t_1 is measured. At the end of a measurement, the data are stored in a two dimensional array, $A(t_1, t_2)$ which is Fourier transformed in both dimensions to give the 2D spectrum (see Figure 2.8).

A rigorous description of 2D methods requires density matrix theory (40). However, a simplified explanation of the COSY experiment can be made to explain how coupling information is encoded into the second dimension.

Consider two coupled protons with different chemical shifts, A and X. In the 1D spectrum each will give a doublet but for simplicity only one peak in the doublet of the A proton, of frequency ν_A , will be looked at. A vector diagram showing the response of this proton magnetization is shown in Figure 2.9. Following the initial 90° pulse the magnetization is tipped into the rotating y' -axis. If a second 90° pulse is applied immediately after (i.e. $t_1 = 0$ seconds) the magnetization is tipped into the $-z$ -axis. If the evolution period is such that the magnetization has precessed into the x' -axis, the second pulse will leave this magnetization unperturbed. Thus, the intensity of the measured magnetization of the A proton oscillates at ν_A (see Figure 2.10). However, if there is coherence transfer between the coupled protons, the amplitude of the

Figure 2.8 Measurement of a COSY spectrum:

- a) FIDs measured at each evolution time, t_1 , stored in matrix $A(t_1, t_2)$,
- b) FIDs Fourier transformed in the t_2 dimension, to give $A(t_1, \omega_2)$,
- c) A contour plot of the array $A(\omega_1, \omega_2)$ following the Fourier transform of $A(t_1, \omega_2)$. This is a COSY spectrum of ovarian cancer cells.

Figure 2.9 Vector diagram showing the response of the precessing magnetization vector, M , to the COSY pulse sequence where a) $t_1 = 0$, i.e., the second pulse is applied immediately after the first pulse and inverts the magnetization, b) during the time $t_1 = t$ the vector has precessed 90° and the application of the second 90° pulse leaves the magnetization unperturbed, c) the vector has precessed 180° and the second 90° pulse returned the magnetization to the equilibrium position d) the magnetization has precessed 270° and is unperturbed by the second 90° pulse and e) the magnetization is again inverted.

Figure 2.10 Variation in the amplitude of the spectral peaks of proton A with changing evolution time t_1 (i.e. the Fourier transformed FIDs as a function of t_1 , the separation time between the pulses). If protons A and X are coupled, the peak amplitudes of A are modulated by the X proton.

resonant peak (in the frequency domain) may in addition be modulated at the frequency of the coupled proton, ν_X .

Thus for a COSY experiment performed on the A nucleus of an AX spin system, two cases should be considered: $J(A,X) = 0$ and $J(A,X) \neq 0$. In both cases, the initial 90° pulse flips the magnetization into the x-y plane. When $J(A,X) = 0$ the second pulse does not affect the information in the x-y plane and the signal is therefore invariant to incremental changes to t_1 (49). This gives rise to the diagonal peaks at (δ_A, δ_A) , where δ_A is the chemical shift of nucleus A. Thus, diagonal peaks result from protons whose frequency either does not change between the evolution and detection period or changes by moving from the resonant position of one line of a multiplet to another.

For the second case where $J(A,X) \neq 0$, the effect of the second pulse is to transform the precession frequency of some of the magnetization vectors precessing at ν_A to ν_X which then precess at ν_X during the detection period. These coupled spins give rise to the off-diagonal or cross-peaks. The frequency coordinates of these cross peaks, (δ_A, δ_X) and (δ_X, δ_A) , indicate the chemical shifts of the coupled partners, i.e. the chemical shifts of the coupled nuclei are correlated.

Similarly to the 1D spectral processing, the 2D spectra were enhanced using filter functions. Either Lorentzian -Gaussian transformations or sinebell weighting functions were used. The optimization of these filter functions for the cells and biopsy samples is discussed in Appendix 1.

2.2 Multivariate Analysis

2.2.0 Introduction

A pattern classifier assigns a "diagnosis" or class to an object (which may contain many attributes). In the context of this work, the object is an MR spectrum from a given sample and the attributes are the intensity values over the frequency range of interest. A classifier will assign a probability that the object belongs to a particular class.

Many classifiers have been used to distinguish between MR spectral characteristics from pathological and normal tissues: neural nets (51), principle component analysis (52), linear discriminant analysis (11,53) No single classifier has been found optimal for all diagnoses. For this study, however, it was found that linear discriminant analysis (LDA) was able to classify ovarian cancer biopsy samples with very high sensitivity and specificity*. This classifier will be discussed in more detail in the following section.

2.2.1 Linear Discriminant Analysis

LDA is a commonly used method for reducing multidimensional data sets to scalar values which can be used to assign each member of the set to a class. This can be described most simply by presenting an example.

Consider two groups with known final outcomes (or diagnoses), positive or negative. Two independent measurements are made on each sample in the

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* Sensitivity is the true positive fraction or the fraction of disease which is found
Specificity is the true negative fraction or the fraction of healthy patients identified

two groups, X_1 and X_2 (54). Scatter plots of these measurements are presented in Figure 2.11. The open circles represent the positive group, the filled circles the negative group. Ellipses can be drawn around each group to contain a given proportion of members. The line between the intersection of the two ellipses is normal to a new axis, which is a linear combination of the variables X_1 and X_2 . Projection of the sample data onto this axis results in each sample being represented by a scalar value. A decision whether to classify a sample as positive or negative can be made on the basis of whether the sample has a value that is higher or lower than a critical value.

To present this more formally, let $x_1, x_2, \dots, x_n$ be n vectors, each with p dimensions, in the data set under study (55,56). In the context of this study each vector would represent the amplitudes of a spectrum measured from a biopsy sample. This set of vectors is comprised of n_1 samples diagnosed as positive (or from class 1) forming a subset X_1 and n_2 samples diagnosed as negative (or from class 2) forming a subset X_2 . The sample means are given by

$$m_i = \frac{1}{n_i} \sum_{x \in X_i} x \quad i = 1, 2 \quad 2.7$$

Let w be the desired discriminant vector and $y_i = w^T x_i$. Define Y_1 and Y_2 such that if $x_i \in X_1$ then $y_i \in Y_1$ and if $x_i \in X_2$ then $y_i \in Y_2$. The means of these subsets are

$$\bar{m}_i = \frac{1}{n_i} \sum_{y \in Y_i} y = \frac{1}{n_i} \sum_{x \in X_i} w^T x = w^T m_i \quad i = 1, 2 \quad 2.8$$

Figure 2.11. Two group discriminant analysis. The dashed line indicates the decision boundary above which a sample is assigned to one group and below which it is assigned to the other group.

The group i covariance matrices for these two subsets are

$$S_i = \sum_{x \in X_i} (x - m_i)(x - m_i)^T \quad i = 1, 2 \quad 2.9$$

and the scatter of the Y_i populations is defined to be

$$\bar{s}_i^2 = \sum_{y \in Y_i} (y - \bar{m}_i)^2 = w^T S_i w \quad i = 1, 2 \quad 2.10$$

One example of a linear discriminant vector is the Fisher Linear Discriminant (57) which is the vector w which maximizes the expression

$$J(w) = \frac{|\bar{m}_1 - \bar{m}_2|}{\bar{s}_1^2 + \bar{s}_2^2} = \frac{|w^T (m_1 - m_2)|}{w^T (S_1 + S_2) w} \quad 2.11$$

If the two subclasses have equal covariance matrices, i.e. $S_1 = S_2$, then $J(w)$ is maximized when

$$w = \frac{(m_1 - m_2)}{S_i} \quad 2.12$$

and thus the separation between the classes is maximized while minimizing the variance within each class. Having established the optimal discriminating vector between the two classes, each x_i can now be associated with the product $y_i = w^T x_i$. Each sample, x_i , can now be reclassified according to a threshold value. The agreement between the class assignment of x_i and y_i gives

a measure of the accuracy of the classification. Thus each p -dimensional vector representing the p attributes of a sample has been transformed into a scalar value which can be interpreted as the probability of a sample belonging to a particular class.

The ability to generalize the performance of a classifier can be evaluated by determining the best discriminant on a training set of objects and then using this discriminant to classify a test set (11). In addition, the training set can be cross validated by using, for example, the leave-one-out (LOO) method. This reduces the amount of bias in estimating the discriminant vector that may accompany small training sets. In short, for a training set with K objects, this method classifies a given object based on the discriminant vector estimated from the remaining $K-1$ objects. This procedure is repeated K times, leaving each object out in turn.

As will be discussed in Chapter 5, samples from tumours are extremely heterogeneous and there may be inter- and intra- observer variability in the diagnoses of the tumours by the pathologists. Thus, there is uncertainty in the assignments made to the training set. One method for dealing with uncertainty is the use of fuzzy sets (11,58) which assigns to each object a measure of membership to a given class. Consider the example from Cios *et al.* (59) for the fuzzy set "tall" (see Table 2.1). A degree of membership to the set tall is assigned to an individual on the basis of height. There are people who are definitely tall (with a degree of membership 1) or definitely not tall (with a degree of membership 0) but, in addition, there are borderline cases with grades of membership between 0 and 1. A person who is not at the extreme ends on a height scale can have partial membership in the fuzzy set "tall"

Table 2.1 Membership in the fuzzy set "tall"

Height (m)	Degree of Membership
<1.52	0.00
1.63	0.08
1.73	0.24
1.82	0.50
1.93	0.76
2.03	0.92
>2.13	1.00

In this work, the degree of membership of a sample to a given class was assigned using the leave-one-out method. This was done as follows: if the training set contained K attributes, one sample was excluded from the classification and the best discriminating vector was determined based on the remaining samples. Each member of the test set was assigned to a class based on this discriminating vector. This process was repeated K times, leaving each sample of the training set out in turn. Thus each sample in the test set was classified K times. The fraction of times a given sample in the test set is assigned to a particular class is a measure of the membership to that class. This gives an estimate to the "fuzziness" of the class assignment.

Identification, and the possible exclusion from the training set, of samples which are too "fuzzy" to classify may be necessary to classify the remaining samples reliably. These samples can be examined using other classifiers or by employing the recently proposed consensus diagnosis strategy (11).

2.3 Sources of Error in the Interpretation of MR Spectra

Biological specimens, be they cells or biopsies, are highly variable and a trade off is made between sample size (i.e. the number of repetitions or the number of specimens) and the time and cost of accumulating large sample sizes.

Sample variations can manifest themselves as changes in peak height, peak width and chemical shift of any given metabolite. A large underlying baseline is also present in the MR spectra which arises from motionally restricted protons (for example protons in membrane lipids as discussed previously), strong solvent peaks or noise. Distortion to this baseline can result from any errors in the measurement of the early part of the FID measurement. Several methods to remove the baseline have been used. Commonly, the spectra are collected within a large frequency window, and the baseline is fit to a cubic spline or polynomial function. A spectrum can also be line broadened (LB ~ 200 Hz) and this line broadened spectrum can be subtracted from the original spectrum. Different choices of baseline correction will lead to changes in the quantitation of peak areas.

Another source of error results from phasing of the spectra. The phase is the angle (in the rotating x'-y' plane) between the starting position of the tipped net magnetization vector and the -y' axis. Adjusting this phase (0th order) is usually done during processing. In practice, the phase of a resonance peak may depend on its frequency and higher order phase correction is often necessary. Variations in the phase corrections will also affect the interpretation of the spectra.

The impact of these later two sources of error (phasing and baseline correction), will be examined on a typical ^1H MR spectrum of cells in Appendix 2. In summary this appendix shows that error in phasing and

baseline correction could contribute to errors in the estimate of the peak height of order 10%.

Shimming imperfections result in the non-exponential decay of the FID resulting in non-Lorentzian lineshapes.

In biological samples there are regional variations in cell density and matrix and differing concentrations of divalent cations (Mg^{2+} , Ca^{2+}) which will broaden lines (magnetic susceptibility effects) differently from one sample to another. These effects result in errors in fitting spectra.

An additional source of error is present for the biopsy study. Significant inter- and intra- observer variability in the histopathological diagnoses of biopsy specimens has been documented (60). These diagnoses are used as the gold standard for our analyses but may in fact be "brass standards".

CHAPTER 3 ^1H MRS OF CULTURED HUMAN OVARIAN CANCER CELLS

3.0 Introduction

Ovarian cancer is often highly heterogeneous. In addition to cancer cells, a tumour specimen may contain immune cells, blood vessels, adjacent normal tissue and areas of necrosis (61). Ideally, to interpret the MR spectra from ovarian cancer, the MR characteristics of each component should be examined individually in an environment similar to what would be found *in vivo*. However, every model of the *in vivo* situation has inherent disadvantages, and a balance must be made between uniformity and relevance (62). Ovarian cancer cells can readily be grown in a culture whereas cells derived from normal ovarian tissue pass through only a few generations and must be 'immortalized' to become established cell lines. The criticism leveled at *in vitro* models is that their responses to stimuli, including drugs and radiation, may not necessarily be representative of their responses in the parental tissue. Animal models, which offer an additional level of complexity, may not adequately reflect the physiology and responses found in humans.

While recognizing these limitations, we chose to examine the ^1H MR characteristics of two cultured human ovarian cancer cell lines derived from untreated patients as a first step towards identifying subpopulations of cancer cells within a heterogeneous sample.

In this chapter, the question of whether cellular changes accompanying the induction of drug resistance are observable by MRS will also be addressed. ^1H spectra of two cisplatin resistant cell lines derived by exposure of the two parental cell lines to cisplatin (63,64) were also measured and compared to

the results obtained from the parental cell lines. These resistant cell lines are known to have elevated levels of glutathione (63, 65-70), decreased drug accumulation (63,67,69,71) and the cell line, 2780CP, has enhanced DNA repair capacity (65). Glutathione is known to be one of the factors responsible for allowing resistant cells to survive treatment with cisplatin. Reduced uptake may either result from changes in an active pump which could affect energy metabolites or could be a consequence of membrane changes which affect passive diffusion and which may be reflected by different levels of phospholipid metabolites.

Breast (72) and lung (73) cancer cells resistant to a different class of drug, adriamycin, have been studied using ^{31}P MRS and increased phosphocreatine levels and decreased (breast) or unchanged (lung) levels of PME_s and PDE_s were observed in comparison to their parental cell lines. Adriamycin is a different class of drug from cisplatin and the mechanisms of resistance to adriamycin probably differ from those responsible for cisplatin resistance. Of the metabolites that can be measured using 1D and 2D COSY ^1H MR Spectroscopy, we hypothesized that glutathione, total choline and total creatine resonances could be used to distinguish resistant from sensitive cell lines. The individual contributions of PCho, GPC, PEth and GPE could be observed using 2D methods.

3.1 Materials and Methods

3.1.0 Cells and Cell Methods

The human ovarian cancer cell line, A2780, was kindly provided by Dr J Gerlach, Ontario Cancer Foundation, Kingston, Ontario. Its cisplatin resistant mutant, 2780^{CP}, was obtained from Dr. Ozols, Fox Chase, Philadelphia Dr Howell, Cancer Center, U.C.S.D., California, generously provided us with two additional human ovarian cancer cell lines (2008, parent; C13*, cisplatin resistant mutant). All cell lines were subcultured twice weekly and were routinely tested for mycoplasma contamination.

Cells were grown at 37°C in Dulbecco's Modified Eagle's Medium and Nutrient Mixture Ham's F12 (DMEM/F-12) (1:1) medium supplemented with 7.5% newborn calf serum and 7.5% foetal bovine serum, 0.1 mM MEM non-essential amino acids, 10 mM sodium bicarbonate and 20mM HEPES in an atmosphere of 98% air/ 2% CO₂. Cells were plated at a density of 4×10^3 cells/cm² and the medium was replaced on day five post-plating. Cells were grown to confluency in standard 25-cm² tissue culture flasks and were harvested on the eighth day post-plating for the drug and radiation survival curves.

Prior to performing the MR experiments, the cisplatin drug response and radiation response curves were measured on these cell lines to confirm that these cell lines had maintained their level of resistance in our hands. For the drug-dose response curves, Cisplatin (Platinol AQ) was administered to the cells at doses varying from 0-20 µg/ml for one hour. The cells were then rinsed three times with isotonic citrate saline, trypsinized (0.2% w/v trypsin in citrate saline for 5 minutes at 37°C) and counted. Counting was performed

by a Elzone 280 Particle Data Cell Counter (Particle Data Inc, Ill). Cell survival was assayed using the colony forming assay. Briefly, this assay entails plating the appropriate number of cells into 60 mm dishes to obtain approximately 50 colonies. A colony was counted if it contained more than 50 cells, 14 days after plating.

Radiation survival was measured by irradiating cells on ice in a Muller X-ray machine at a dose rate of 112 cGy/min. Survival was determined using the colony forming assay as described for the cisplatin survival studies above.

For the MRS experiments, cells (5×10^7) were harvested in plateau phase from large tissue culture dishes (22.5cm x 22.5 cm), rinsed three times with phosphate buffered saline in deuterium oxide (PBS/D₂O) and resuspended in 0.4 ml of the same buffer. The cell suspension was transferred to a 5 mm NMR tube.

Depletion of cellular glutathione in the cisplatin resistant cell line, C13*, was accomplished by administering 25-50 μ M of L-buthionine sulfoximine (BSO) for 24 hours prior to harvesting them. BSO is a synthetic amino acid which irreversibly inhibits the enzyme γ -glutamylcysteine synthetase.

Assignment of glutathione resonances was made by measuring the 1D MR spectrum of a 5mM glutathione solution in PBS/D₂O.

Chemicals and cell culture supplies were obtained from Sigma, Mississauga, Ont.

3.1.1 MR Methods

With the exception of the glutathione depletion studies, all ¹H MRS measurements were performed at 37° C on a Bruker AM360 (360 MHz) wide

bore spectrometer using a standard 5mm probe head. The glutathione depletion studies were performed on a Bruker narrow bore 500 MHz spectrometer. 1D spectra were acquired following a 90° pulse with a spectral width of 5000 Hz, 640 accumulations, 4K data points and a 2.41 s repetition rate. Water suppression was accomplished using selective gated irradiation. p-Amino-benzoic acid (PABA, 10 mM) in a capillary was used as a chemical shift and integration standard. A 2 Hz line broadening was applied prior to Fourier transformation and 1D spectra were processed using standard Bruker software and MACFid (TECmag, Houston, TX). Magnitude mode COSY spectra were acquired with 90° pulses, a 1.36s repetition time, a spectral width of 2824 Hz in the F2 domain and 32 transients. In the t_2 time domain, 2K points were collected and in the t_1 time domain 160 FIDs were acquired. COSY spectra were processed to ensure that short T_2 components could be observed (74). Details on the parameters chosen for COSY processing can be found in Appendix 1. Briefly, sinebell window functions were applied in both dimensions (TD2=512 Words (W), TD1=160W). COSY matrices were zero filled in t_1 , Fourier transformed in both dimensions, symmetrized and presented as magnitude spectra.

Following the measurement of the 1D spectrum and the COSY, a second 1D measurement was obtained to ensure that no significant changes to the spectrum occurred during the COSY measurement. Cell viability, as determined by the trypan blue exclusion test (trypan blue 0.4% w/v) was greater than 85% for the duration of the MR experiments. To compare cell lines, the number of cells used for the MR measurements was kept constant and spectra were scaled with respect to the standard (10mM PABA). Peak assignments to the resonances in the 1D spectra were made using the COSY

spectra, by addition of standard compounds and by comparison with published chemical shift values (8,75,76).

3.1.2 Statistical Analysis

MR and survival measurements were repeated on each cell line three to four times. MR data are presented as mean $\pm$ one standard deviation in tables and survival measurements are presented as mean $\pm$ one standard error of the mean. Means were compared using the unpaired t-test. Linearized radiation survival curves were fitted to second order polynomials using Grapher 1.24 (Golden Software, Inc., Golden, CO).

3.2 Results

3.2.0 Biological Characteristics

Prior to comparing the ^1H MR spectra of these cell lines, key biological characteristics were measured. Population doubling time, cell plating efficiency and the drug and radiation doses required to kill 50% of the cells were determined. This was done to ensure that, in our hands, the response of cells to drug and radiation treatment was preserved. These characteristics are defined below.

The growth curve of the A2780 ovarian cancer cell line is shown in Figure 3.1. This curve exhibits the standard sigmoid shape. The population doubling time for each curve was determined for the growth in the exponential region of the curve (between 1 and 5 days post plating).

The curves were fit to

$$\text{Cell \#} = N = N_0 \exp\left(\frac{t}{T_d}\right)$$

3.1a

Figure 3.1 Growth curve of the human ovarian cancer cell line, A2780 grown at 37°C in DMEM/F-12. The medium was replaced on day five for plates indicated by the closed circles.

Table 3.1 Biological characteristics of four human ovarian cancer cell lines.

Biological Characteristics	A2780	2780CP	2008	C13*
Plating Efficiency (%)	28.4 ± 4.9	35.3 ± 9.1	31.6 ± 3.7	33.8 ± 1.9
Doubling Time (hr)	17.4 ± 1.5	17.4 ± 1.2	20.3 ± 2.6	21.5 ± 4.3
Cell Density (10 ⁵ cells/cm ²)	6.7 ± 1.3	6.9 ± 0.4	4.9 ± 0.4	4.9 ± 0.9
α (Gy ⁻¹)	1.02 ± 0.16	0.61 ± 0.31	not measured	not measured
β (Gy ⁻²)	0.03 ± 0.02	0.02 ± 0.02		
Fold radiation resistance ¹	1	2.4	-	-
ID ₅₀ radiation (Gy)	0.97 ± 0.05	2.35 ± 0.12	-	-
Fold cisplatin resistance ¹	1.0	7.1	1.0	19.4
IC ₅₀ cisplatin (μg/ml)	1.3 ± 1.0	9.2 ± 2.3	0.8 ± 0.4	17.5 ± 1.7

Values measured in our laboratory on plateau phase cells.

¹ Fold resistance is the ratio of dose to achieve 50% survival (ID₅₀ for radiation, IC₅₀ for cisplatin) of a cell line compared to the parental cell line.

and the population doubling time, T_d , is

$$T_d = (\ln 2) (T_2) \quad 3.1b$$

The doubling times (averaged over three growth curves per cell line) are presented in Table 3.1. The cell densities in plateau phase following a media change on the fifth day post-plating are also given. Beyond ten days, the cell numbers started to decline. For the MRS experiments, cells were harvested on day eight after a day five feeding.

The cell plating efficiency, PE, is defined as

$$PE = \frac{\# \text{ colonies counted}}{\# \text{ cells plated}} \quad 3.2$$

The PE values for each cell line can be found in Table 3.1. PE values of untreated cells were determined for every survival experiment. This PE was used to normalize the surviving fraction of untreated cells to one. The survival fraction, $S(D)$, is defined as

$$S(D) = \frac{\# \text{ colonies counted}}{(\# \text{ cells exposed to } D \text{ and plated})(PE)} \quad 3.3$$

where D is the dose of radiation (for the radiation survival curves) or the dose of cisplatin (for the drug survival curves)

The linear quadratic model (77) is a widely accepted model of radiation dose response and incorporates two components of cell killing: the formation of double strand breaks either by a single event or by two events. This model is expressed as

$$S(D) = \exp(-\alpha D - \beta D^2) \quad 3.4$$

Individual radiation survival curves of two of the cell lines (A2780 and 2780^{CP}) measured previously in the laboratory were fitted to a linearized form of Equation 3.4. The curve fitting routine fitted the natural log of the survival data to a second order polynomial. Fits were made to each individual survival curve. Fitted radiation curves are shown in Figure 3.2 and the estimated values for α , β and the ID_{50} (dose required to kill 50% of the cells) for both cell lines are given in Table 3.1. At this level of killing, the cisplatin resistant cell line is 2.4-fold resistant to radiation.

Similarly, the survival of two cell lines (A2780 and 2780^{CP}) exposed in plateau phase to increasing doses of cisplatin is presented in Figure 3.3. The logarithms of these curves were also fitted to a second order polynomial. However, as the mechanism of cell killing is not well understood for cisplatin, the linear-quadratic model does not correspond to an underlying biological model.

The cisplatin IC_{50} for each cell line is the dose required to achieve an $S(D) = 0.5$ and can be found in Table 3.1. The 2780^{CP} cell line is seven-fold resistant compared to its parental cell line and the C13* cell line is twenty-fold resistant.

Previously published biological characteristics were measured on logarithmically growing cells. However, our measured characteristics (drug survival, radiation survival, doubling time and plating efficiency) were done on plateau phase cells and thus these biological measurements have not been published elsewhere.

Figure 3.2 Radiation survival curves of two human ovarian cancer cell lines, A2780 and 2780^{CP}. These curves were fitted using the Linear Quadratic Model.

Figure 3.3 Drug response curves of two human ovarian cancer cell lines, A2780 and 2780CP, exposed to the anti-cancer drug cisplatin.

3.2.1 Magnetic Resonance Spectra of Human Ovarian Cancer Cell Lines

A typical 1D MR spectrum of the 2008 cell line is shown in Figure 3.4. Metabolites contributing to the 1D spectra were assigned using the 2D COSY spectra (see following section), addition of standard compounds and published values (75,76). Many of the peaks have contributions from several metabolites. Only assignments of the major contributors are shown

Establishing the Reproducibility of the MR Measurement

Proton spectra from four separate preparations of the same cell line (C13*) are shown in Figure 3.5. Compared to the variations seen between spectra from the four different cell lines (Figure 3.6) only small variations in the metabolic profile are evident between the spectra of the same cell line

General Features of the Four Human Ovarian Cancer Cell Lines

Spectra from the two parental human ovarian cancer cell lines (A2780 and 2008) and their treatment resistant mutants (2780CP and C13* respectively) are shown in Figure 3.6. Prominent resonances in the 1D spectra from all four cell lines include methyl (0.9 ppm), methylene (1.3 ppm), total choline which contributes to the broad peak at 3.2 ppm, total creatine (3.0 ppm) and the olefinic resonance at 5.26 ppm. Also contributing to the spectra in the poorly resolved region between 3.3 ppm and 4.5 ppm are some soluble membrane components, amino acids and other metabolites (8,75,76).

Figure 3.4 A typical 1D spectrum of the 2008 cell line

Figure 3.5 ^1H MR spectra from four separate preparations of the cisplatin resistant ovarian cancer cell line C13*

Figure 3.6 ^1H MR spectra from four ovarian cancer cell lines; two drug-sensitive parental cell lines (A2780 and 2008) and their cisplatin-resistant mutants (2780^{cp} and C13*).

Comparison of the 1D ^1H Spectra from the 2 Sensitive Cell Lines.

The 2008 cell line has a greater number of well resolved peaks including the resonances from choline and glutathione or glutamine which are more intense than in the A2780 cell line (see Tables 3.2 and Figure 3.6). The olefinic resonance at 5.26 ppm is greater in the A2780 cell line.

Comparison of the Drug -Sensitive and -Resistant Cell Lines - 1D MRS

The key goal of this study was to determine whether there were any spectral differences between drug -sensitive and -resistant cell lines. Prior to comparing these cell lines, the quantification of the spectral features of these cell lines will be briefly discussed.

The area under each resonance peak is proportional to the concentrations of the species contributing to it. These spectra are extremely complex and many of the peaks are poorly resolved and may have several metabolites contributing to them. Some of this degeneracy is overcome by 2D methods but quantification of COSY spectra is also not very accurate.

Several methods of quantification are routinely used (78). Firstly, if the peaks were truly Lorentzian, the peak area can be calculated from the peak height and width at half maximum. If one assumes that the line width remains constant, then the area of the peak is proportional to the peak height. Comparison of peak heights was used by MacKinnon *et al.* (31), Delikatny *et al.* (13) and Kuesel *et al.* (30). Secondly, spectra can be integrated between a range of chemical shifts. With overlapping signals and the problem of the wings in the Lorentzian line shape extending to infinity, this technique also only gives rough estimates of the area. It has, however, been used successfully

to distinguish between different grades of cervical cancer and brain neoplasms (9,10). A third method is to fit the spectra to a sum of Lorentzian line shapes. With this approach the shape of the resonance peak is assumed to be Lorentzian or is converted to a Gaussian using the Lorentzian-Gaussian conversion and the number of peaks used to fit a region with overlapping resonances is arbitrary. This approach has been primarily employed on spectra from brain tissue which does not contain the large lipid spectrum seen in normal and pathological ovarian tissue. Quantification of metabolites can also be done by fitting the FID in the time domain (78). A spectrum of the A2780 cell line is fitted to Lorentzian line shapes in Appendix 3. The same set of Lorentzian peaks could not be used to fit the sensitive/resistant pair and the difference between the fitted peak spectrum and the measured spectrum had a significant amount of signal remaining. Thus, for this work, peak heights and integrated peak areas were measured. By measuring peak amplitudes, our work can be directly compared to published cell MR measurements and can also be more easily compared with the results from the pattern recognition analyses of biopsy samples found in Chapter 5. Having identified peak amplitude differences between the sensitive and resistant pairs, these peaks were integrated between the chemical shift ranges shown in Table 3.4. This allows our studies to be directly compared with studies by Rutter *et al.* (9) and Kuesel *et al.* (10).

The spectra from each pair found in Figure 3.6 are replotted in Figure 3.7 to emphasize the difference between cell lines in each pair. The sensitive parental lines are drawn with solid lines and the resistant mutants are overlain with dashed lines. Differences between the pairs are indicated by dashes. The peak amplitudes of these resonances were measured relative to the reference and are presented in Table 3.2.

Figure 3.7 Spectra of four ovarian cancer cell lines (seen in Figure 3.6) replotted to emphasize the differences between the cell lines in each pair

Table 3.2. Relative peak amplitudes[#] of selected peaks from four ovarian cancer cell lines

Chemical Shift (ppm)	Metabolites	2008 (n=4)	C13* (n=4)
3.93	Creatines	0.42 ± 0.10 [‡]	0.32 ± 0.02
3.77	Glutamate/ Glutamine/ Glutathione	0.25 ± 0.07 [‡]	0.50 ± 0.02
3.42	Taurine	0.04 ± 0.02	0.11 ± 0.04
3.28		0.28 ± 0.16 [‡]	0.20 ± 0.02
3.03	Creatines	0.68 ± 0.18 [‡]	0.59 ± 0.05
2.55	Glutamine/ Glutathione	0.09 ± 0.03 [‡]	0.35 ± 0.14
2.24		0.38 ± 0.11	0.48 ± 0.12
2.16	Glutamine/ Glutathione	0.35 ± 0.05	0.64 ± 0.10
1.72	Lysine	0.41 ± 0.16	0.43 ± 0.08
1.58		0.37 ± 0.14	0.58 ± 0.16
1.30	(CH ₂) _n	1.33 ± 0.35	0.91 ± 0.24
Chemical Shift (ppm)	Metabolite	A2780 (n=4)	2780cp (n=4)
3.23	Cholines	1.16 ± 0.45 [‡]	0.40 ± 0.12
3.03	Creatines	0.54 ± 0.11	0.36 ± 0.10
2.34	Glutamate	0.50 ± 0.08	0.29 ± 0.07
2.09	Glutamate	1.35 ± 0.16	1.03 ± 0.14
1.31	-CH ₂ -	1.62 ± 0.08	1.68 ± 0.08

[#] Peak amplitude relative to the reference peak. Data presented as mean ± one standard deviation. Shaded areas indicate that the means of the pair are significantly different using the two-sided student t-test (P<0.05).

[‡] Alternate t-test used which does not assume equal variance.

Four significantly different regions were found between the 2008 and C13* (shaded in Table 3.2), three of which (3.77, 2.55, 2.16 ppm) could not be unambiguously assigned. Two candidates for these resonances are glutathione and glutamine. Elevated glutathione levels have been observed in resistant cells whereas glutamine has not been implicated as part of a mechanism for conferring resistance to cells. It was therefore important to establish whether the elevated peaks were primarily from glutathione. A high resolution 1D spectrum of glutathione was measured in a Bruker AM500. This spectrum and the molecular formula for glutathione are shown in Appendix 4. Cellular glutathione levels of the resistant mutant were reduced by exposing the C13* cell line to 25 and 50 μ M BSO for 24 hr prior to the MR measurements. Proton spectra from these preparations are shown in Figure 3.8. The arrows indicate the positions of the glutathione peaks. The amplitude of the glutathione peak at 2.55 ppm decreased to 40% and 32% of the untreated level following exposure to 25 and 50 μ M BSO respectively. This latter reduced intensity was within the range observed in the parental cell line.

Three significantly different regions were found between the A2780 and 2780^{CP} cell lines. These were the composite choline peak and the two glutamate peaks.

The integrated resonances of glutathione and taurine from the 2008 and C13* cell lines are found in Table 3.3. In agreement with the peak height measurements, the C13* cell line had a four-fold increase in the glutathione resonance at 2.55 ppm and a two-fold increase in the taurine resonance compared to the 2008 cell line. Also in Table 3.3 are the integrated areas from the A2780 and 2780^{CP} cell lines of choline and glutamate.

Figure 3.8 ^1H spectra from the resistant ovarian cancer cell line C13*

a) with no exposure to BSO, b) exposure to 25 μM BSO for 24 hours prior to MR measurement and c) exposure to 50 μM BSO for 24 hours prior to MR measurement

Hence the observed differences between the 2008 and C13* cell lines were in the levels of glutathione and taurine. Differences observed between the A2780 and 2780^{CP} cell lines were in the glutamate regions. The mean integrated choline resonance of the A2780 cell line had a large error associated with it and the difference in this resonance between the parent/mutant pair was found not to be significant.

Table 3.3. Relative peak areas[#] of selected peaks from four ovarian cancer cell lines

Chemical Shift (ppm)	Metabolite	2008 (n=4)	C13* (n=4)
3.74 - 3.82	Glutathione	1.01 ± 0.11	1.39 ± 0.12
3.38 - 3.48	Taurine	0.11 ± 0.05	0.20 ± 0.05
2.50 - 2.60	Glutathione	0.23 ± 0.08	0.95 ± 0.04
Chemical Shift (ppm)	Metabolite	A2780 (n=4)	2780 ^{CP} (n=4)
3.40 - 3.08	Choline	2.81 ± 1.30	1.27 ± 0.33
2.45 - 2.32	Glutamate	0.89 ± 0.06	0.51 ± 0.13
2.16 - 1.90	Glutamate	5.84 ± 0.71	4.07 ± 0.55

[#] Peak areas relative to the reference peak.

Data presented as mean ± 1 standard deviation. Shaded areas indicate that the means of the pair are significantly different using the t test (P<0.05)

Comparison of the Drug -Sensitive and -Resistant Cell Lines - 2D MRS

In addition to intense cross peaks from the fatty acid chains of triglycerides (see Appendix 5), the 2D COSY spectra of the four cell lines (seen in Figures 3.9, 3.10 and Table 3.4) have prominent cross peaks from numerous other

Figure 3.9 ^1H 2D COSY spectra of the sensitive and resistant ovarian cancer cell line pair A2780 and 2780CP.

Figure 3.10 ¹H 2D COSY spectra of the sensitive and resistant ovarian cancer cell line pair 2008 and C13*

Table 3.4 Identification of metabolites observed in 2D COSY spectra from four ovarian cancer cell lines.

Metabolite	Abbr.	Chemical Shift F2-F1 (ppm)	2008	C13*	A2780	2780CP
<u>Amino Acids and related Compounds</u>						
Taurine	Tau	3.28 - 3.44	s	y	y	y
Glutamate	Glu	2.09 - 2.34	y	y	y	y
Glutathione/ Glutamine	GSH/ Gln	2.15 - 2.55	s	y	n	s
Glutathione/ Glutamine/ Glutamate	GSH/ Gln/ Glu	2.15 - 3.78	n	y	s	s
Isoleucine	Ile	0.97 - 2.12	-	-	y	y
Lysine	Lys	1.72 - 3.05	y	y	y	y
<u>Membrane Related Compounds</u>						
Triglyceride	A	0.90 - 1.30	y	y	y	y
	B	1.33 - 2.08	y	y	y	y
	E	1.33 - 1.62	y	y	y	y
	F	1.60 - 2.30	y	y	y	y
Phosphorylcholine	PCho	3.61 - 4.25	n	y	y	n
GlyceroPC	GPC	3.69 - 4.38	n	s	n	s
Inositol	Inos	3.28 - 3.64	-	-	y	y
<u>Other Compounds</u>						
Lactate Anion	Lac	1.30 - 4.12	s	s	y	y
HEPES		3.15 - 3.90	y	y	y	y

Differences observed between parental cell line and mutant

- y Strong cross peak intensity
- n Cross peak not present
- s Cross peak present with low intensity

metabolites including glutamate, taurine, inositol and choline compounds. Couplings and assignments for the observed metabolites are given in Appendix 4. Initially the cell lines were compared visually. Each of the cross peaks was assigned to one of three bins: no observable intensity (n), low intensity (s) and high intensity (y). These assignments are shown in Table 3.5 and the significant differences observed between the sensitive and resistant cell lines are given in the shaded boxes.

To quantify the observed differences, volumes of selected cross peaks were obtained and are presented in Table 3.5. The window function chosen to process the spectra enhances cross peaks in a manner dependent on their T_2 values (48,74) and hence caution must be taken in comparing different cross peak volumes within a spectrum. These spectra were, however, all processed identically and differences in a given cross peak volume compared to that measured in another cell line can be compared. The glutathione peak was found to be 2.1-fold higher in the C13* cell line compared to its sensitive parent. The taurine level was 1.8-fold increased. The resistant cell line, 2780cp, had a 1.6-fold increase in the amplitude of the GPC cross peak. No other peaks were found to be significantly different between the wild type/ mutant pairs.

Table 3.5 Volumes of selected metabolites of four ovarian cancer cell lines[§]

Metabolite	2008 (n=)	C13* (n=4)	A2780 (n=4)	2780cp (n=4)
<u>Amino Acids and related compounds:</u>				
Taurine	41 ± 6	75 ± 5	48 ± 10	64 ± 13
Glutathione/ Gln	8 ± 3	17 ± 4	7 ± 1	11 ± 4
<u>Membrane Related Compounds</u>				
Phosphoryl-ethanolamine	16 ± 4	28 ± 5	18 ± 1	17 ± 4
Choline	9 ± 3	13 ± 10	10 ± 1	14 ± 5
Phosphorylcholine	16 ± 3	25 ± 5	18 ± 10	15 ± 10
Glycero PC	7 ± 5	7 ± 2	7 ± 2	11 ± 2

Shading indicates that the wild type/ mutant pair are significantly different ($p < 0.05$) using the 2 tailed p test.

* Ratio of PCho/ PEth calculated from individual measurements and averaged over the measurements made on each cell line.

§ Volumes relative to the cross peak volume of the reference.

3.3 Discussion

The two ovarian cancer parental cell lines are a model of intrinsically sensitive ovarian cancer cells within a tumour. The two resistant mutant cell lines model cells which have acquired resistance to cisplatin and, in one cell line, radiation. The 2008 cell line was established from a patient with serous cystadenocarcinoma of the ovary (67). The mutant cell line was developed by monthly exposures to cisplatin, followed by chronic exposure to stepwise increasing concentrations of the drug (63). The A2780 cell line was derived from a patient with untreated ovarian cancer (64). The cisplatin resistant mutant, 2780^{CP} was developed by intermittent exposure of the parental line to stepwise increasing concentrations of cisplatin (64). The drug exposure schedule used to induce resistance *in vitro* has a large impact on which of the mechanisms of resistance predominate. Chronic exposure to increasing concentrations of cisplatin leads to elevated levels of glutathione. Changes in drug accumulation tend to result from monthly exposure to the drug (79). Clinically, patients are usually administered cisplatin monthly at serum concentrations ranging from 1 to 50 μM (80). It is not known which induction schedule results in a model system which is most relevant to resistance induced *in vivo*. However, the pattern of cross resistance to unrelated drugs and irradiation in clinical ovarian cancer is also exhibited by the 2780^{CP} cell line.

Previously published biological characteristics have been measured on logarithmically growing cells whereas our results were measured on plateau phase cells. It was therefore necessary to ensure that these cells had maintained their level of resistance under these growth conditions. Published values for the IC_{50} of A2780 vary from 0.11-0.91 $\mu\text{g/ml}$ (65,68, 81,82). Given

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the wide range of values and different growing conditions under which survival was measured, our value of 1.3 $\mu\text{g/ml}$ shows the same order of survival. The range published for the 2780^{CF}, 2008 and C13* cell lines are 2.42-12.12 $\mu\text{g/ml}$ (65,81,82), 0.24-1.54 $\mu\text{g/ml}$ (67, 71) and 15.9-34.8 $\mu\text{g/ml}$ (67,71) and our IC₅₀s are within these ranges. Thus, with our growing conditions, the resistant cell lines maintained their level of resistance to cisplatin.

The radiation survival curve of the resistant cell line, 2780^{CF}, had a pronounced shoulder compared to the parental cell line. This shoulder indicates that this cell line is able to accumulate sublethal damage. This cell line is known to have increased DNA repair capacity (22,66) which is one of the mechanisms responsible for resistance.

Differences between sensitive and resistant ovarian cancer cells could be detected by ¹H MRS. Elevated levels of taurine were observed in the cisplatin resistant cell line, C13*, compared to its sensitive parent. Taurine is a sulphur containing β -amino acid that is abundant in tissues like the liver, brain and retina which produce oxidants and toxic substances (83). Taurine is not incorporated into proteins. In the literature, no role for taurine has been proposed for conferring resistance against chemotherapeutic agents on cells. However, there is evidence that taurine does have a protective role on other cells within the body. Taurine may serve as a conjugator and detoxifier of several toxic compounds, as an oxidative scavenger and may assist in membrane stabilization in cells. These roles for taurine are discussed in detail by Zelikovic *et al.* (83). It is not implausible to believe that these protective roles may be important for cells to resist damage caused by therapeutic agents.

The C13* cell line has reduced drug uptake compared to its sensitive parent (67). This could result from some change in the membrane structure or

composition or the presence of an active pump for effluxing the drug. Arguments for the passive diffusion of cisplatin into cells are that a) cisplatin uptake is not saturable and b) it is not inhibited by structural analogs (84). However there is also evidence for protein mediated transport including the observation that activation of some intracellular signal transduction pathways and some non-membrane permeabilizing agents modulate uptake as well (84).

Some investigators (see Mann *et al.* (85) for review) have shown that decreased fluidity can reduce uptake. There is disagreement in the literature, however, regarding the differences in membrane fluidity between the 2008 and C13* cell lines. Using the technique of fluorescence polarization, Mann *et al.* (85) concluded that reduced cisplatin accumulation was not due to decreased membrane fluidity. In contrast, Popovich *et al.* (86) found using pressure tuning infrared spectroscopy on the same cell lines that membrane fluidity of the cell lines was decreased.

The ratio of phospholipids, phosphatidylcholine/phosphatidylethanolamine is used as a measure of fluidity (85) and increased fluidity is associated with increased drug uptake. The phosphorylation of choline and ethanolamine to PCho/PEth is the first step in the biosynthesis of these phospholipids. Hence changes in the ratio of PCho/PEth may reflect changes in membrane biosynthesis. Our results show no significant differences in the phospholipid precursors.

Studies on the membrane stabilizing potential of taurine (87,88) suggest that taurine may mediate its protective effect against cell swelling, lysis and death through an action on membrane permeability and ion transport. A possible interpretation of our results is that reduced passive uptake is not a result of changes in membrane composition, but may exploit the role of taurine

as a membrane stabilizer. Further work needs to be done to clarify this (see Chapter 7 for future directions).

Glutathione is an important detoxifying agent in ovarian cancer. Elevated levels of glutathione have been measured in these resistant cell lines (65,66,68,69). Mechanisms of resistance in these cell lines are multifaceted and elevated glutathione levels are not thought to be the most important mechanism. In addition, MR is not the best technique for measuring glutathione levels (not all the GSH is visible by MRS, total GSH content can be assayed by the method of Griffiths *et al.* (89)). However, MR was able to detect the differences in glutathione content between two cell lines. Hence it may be possible to detect whether ovarian cancer biopsies have high glutathione levels or whether, due to tumour heterogeneity, there is variation in glutathione levels across ovarian cancers. High levels of glutathione observed in ovarian cancer may indicate that a different treatment strategy should be employed or that, in these patients, pretreatment with BSO may improve survival.

An increased level of GPC was observed in the 2780CP cell line, in agreement with our results in Chapter 6. GPC is a degradation product of phosphatidylcholine (90) and increased phospholipid turnover may result from differences in phospholipase activity.

These cell lines were grown in well controlled conditions and, within the same cell line, gave reproducible spectra. Spectral features that the two parental cell lines had in common were large lipid, choline and total creatine resonances. Relative proportions of other metabolites observed in these spectra were different. Thus, one can make a tentative prediction that there may be large variability between spectra from ovarian cancer cell lines derived

from different patients, and biopsy samples which contain many components in addition to cancer cells, will presumably exhibit even greater variability.

3.4 Conclusions

This study showed that differences between sensitive and resistant ovarian cancer cells were detectable by ^1H MRS. One resistant cell line had increased taurine and glutathione and the other increased GPC and decreased glutamate compared to their respective parental cell lines. These results will be used to help interpret spectroscopic data measured on ovarian cancer biopsies from patients who have not responded to therapy.

The two sensitive cell lines were characterized by large lipid, choline and total creatine resonances. These whole cell models provide an intermediate step between extracted cells and tissues on the one hand, and biopsy samples and *in vivo* measurements on the other. All the MR measurements were done on cells which had reached confluency. Ovarian cancers in patients will have areas of exponentially growing cells and plateau phase cells. Differences between cells grown to different densities will be discussed in the following chapter.

Also the sample-to-sample variations that were observed with these cell preparations (grown under well controlled conditions) will be amplified in biopsy samples by the presence of non-cancer cells and possibly by the variable growing conditions found *in vivo*. It was felt that more powerful analyses would have to be deployed. These multivariate techniques will be discussed in Chapter 6.

CHAPTER 4   COMPARISON OF ^1H MR SPECTRA FROM OVARIAN CANCER CELLS IN VARIOUS GROWTH PHASES

4.0   Introduction

Solid tumours tend to grow next to blood vessels. Cancer cells close to the vessel are well supplied with O_2 and nutrients. As they diffuse through the tumour, these nutrients are depleted. Thus there is a nutrient gradient throughout the tumour and, because subpopulations of cells are exposed to different micro-environments, there is a heterogeneous population of cells. A fraction of cells may be exponentially growing whereas other cells may be quiescent. Areas of necrosis develop when cells are not supplied with sufficient nutrients. Thus, without even considering the non-cancer components of biopsy samples, there exists a heterogeneous population of cancer cells in various growth phases and experiencing different growing conditions.

Differences in the biochemical properties of exponentially growing and plateau phase cells are well documented. In particular the drug and radiation response of cells is highly dependent on growth phase (19) and cell-cell contact (91). Since ^1H MR spectroscopy provides a window into the biochemical properties of cells in tumours, differences in these properties may manifest themselves in spectra of cells at different phases of growth. For ^1H MRS to have a role to play in diagnosing and evaluating the treatment sensitivity of ovarian cancer, it is essential to characterize ovarian cancer cells grown under some of the different conditions that may be present *in vivo*.

There are few studies investigating ^1H spectra from whole cells in different growth phases (92,93). In a recent study (92), the lipid spectrum from transformed murine fibroblast L cells could be manipulated by changing the cell growth conditions; elevated lipids could be induced by cell confluence or low pH and further enhanced by serum free culture conditions. In this work, changes in choline metabolites and glutamine were also observed with different culture conditions.

As will be discussed in Chapter 5, the intensity of the choline and lipid peaks in ^1H MR spectra will, in part, be used to discriminate between normal ovarian tissue and malignant ovarian cancer and the glu/gln resonance at 3.7 ppm will be used to differentiate between untreated and recurrent ovarian cancer. Thus, three of the resonances that are useful in classifying ovarian cancer (Chapter 5) are influenced by growing conditions. It is therefore important to gain some insight into conditions which modulate these resonances. Choline metabolites (GPC and PCho) are associated with phospholipid synthesis and degradation (94) and changes in glutamine (and glutamate) levels may reflect changes in energy metabolisms (76). However, the source of the intense lipid signal remains a subject of controversy.

High resolution lipid signals have been observed in tumours arising from the brain (10), cervix (12,13), colon (32) and muscle (95) and from human cancer cell lines (8,96). It has been proposed (6,92) that the malignant transformation of cells is associated with changes in cellular biochemistry which results in changes in the high resolution spectrum. An increased number of MR visible molecules is observed in colorectal cell lines as cells lose their capacity to differentiate (6,8).

To give rise to a high resolution spectrum, these lipids are probably not organized into membrane bilayers, yet they are thought to be associated with

the plasma membrane because the addition of Gd^{3+} (a paramagnetic agent which preferentially binds to the plasma membrane) results in perturbations to these resonances (97). To explain these results a model was proposed in which domains of neutral lipid (predominantly triglyceride and to a lesser degree cholesterol) were able to isotropically tumble within the plasma membrane of malignant cells (98). A second source of the lipid signal was suggested by the results of a study of a murine spleen myeloma cell line in which the presence of mobile lipids correlated with the presence of cytoplasmic droplets (99). May *et al.* (100) found, using stimulated neutrophils, that while the presence of cytoplasmic droplets could account for some of the observed lipid signal, the majority of the signal was associated with the plasma membrane. The source of the high resolution lipid signal in cells may therefore be cell line dependent.

More relevant to our biopsy study in Chapter 5 is the finding that in *ex vivo* biopsy samples from brain astrocytomas, increased amounts of mobile lipid (i.e. MR visible lipid) were found to be associated with the presence of necrotic regions (10,30). These necrotic areas develop in areas of the tumour which have outgrown their nutrient supply. It was hypothesized that the source of these MR visible lipids may be from the breakdown of the plasma membrane.

In the previous chapter, suspensions of single cells were examined. These cells were cultured under uniform conditions and were harvested in plateau phase. Proliferating cells can be modelled by cells growing exponentially in a monolayer. A step closer to the molecular environment found *in vivo* can be achieved using the spheroid model. Spheroids are spherical aggregates of cells having tissue-like density (see Figure 4.1). As in solid tumours, there is a nutrient gradient from the periphery of the spheroid to the centre. Spheroids

have an outer rim of viable cells which, in larger spheroids, encloses a necrotic core (91). The viable rim can further be divided into an outer rim which contains proliferating cells and an inner rim which contains quiescent cells.

Spheroids have been examined extensively using ^{31}P MRS (101,102). These studies have concentrated on energy and membrane metabolism and response to therapy. In addition, several ^1H MR microscopy studies have been published (103). We have employed the spheroid model to determine the effect of cell-cell contact, heterogeneous cell populations and the presence of a necrotic fraction on ^1H MR spectra.

To evaluate the effect of cell cycle, cell density and the accompanying change in the external media conditions on the MR spectrum, cells were grown in flasks at low and high cell densities. At higher cell densities the culture medium had reduced pH and lower nutrient levels. Spectra from cells at the different cell densities were compared. Monolayer exponential cells were then compared to small spheroids ($< 100\mu\text{m}$). These cells, unlike exponentially growing cells grown on tissue culture dishes, are at tissue like densities. Larger spheroids, with heterogeneous populations of exponential and plateau phase cells, were also examined. These spheroids provide an additional step towards interpreting MR spectra from heterogeneous ovarian cancer biopsy samples. Finally, spectra from large spheroids were obtained to characterize the effect of necrotic areas on MR spectra.

Figure 4.1 Typical 650 μm spheroid. Panel A : scanning electron micrograph, X 100 magnification. Panel B: Haematoxylin and eosin stained cross section, X 100 magnification (reprinted from reference 104)

With these models, the human ovarian cancer studies from Chapter 3 can be extended. The three aims of this chapter are to use ^1H MRS to examine: a) exponential versus plateau phase cells, b) exponential cells at tissue-like density and c) the effect of an increasing percentage of necrotic cells. Understanding factors which may influence the MR spectra from normal and pathological ovarian tissue may improve the interpretation and diagnostic ability of ^1H MR spectroscopy.

4.1 Materials and Methods

4.1.0 Cell Methods

Monolayers: The human ovarian cancer cell line used in this study, A2780, has been discussed in Section 3.1.0. Cells were grown as before but were harvested three days (early exponential) or eight days (confluent) after plating. Media was replaced on day five for the cells grown to confluency.

Spheroids: For spheroid growth, cells were harvested during exponential growth, seeded into petri dishes at a density of 10^4 cells/ml medium for 5 days and transferred to a 500 ml spinner flask containing 300 ml of DMEM/F-12 tissue culture medium with the addition of penicillin/ streptomycin. The spinner flask was placed in a cabinet maintained at 37°C on a magnetic stirrer. The magnetic stir bar in the spinner flask (Figure 4.2) rotated at 120 rpm. The air cavity was flushed with 95% air / 5% CO_2 . Media (250 ml) was replaced every two days for two weeks, and daily for the remaining growing period.

Figure 4.2 Spheroids growing in a spinner flask with a magnetic stirrer rotating at 120 rpm.

Exponential vs. plateau phase cultures: ^1H MR spectra from monolayer confluent cells (day eight) were compared to cells growing exponentially in monolayers (day three) or as spheroids. Small spheroids ($<100\text{ }\mu\text{m}$) were removed eight days after initial seeding into petri dishes. The pH of the medium immediately prior to cell harvest was also measured.

Spheroids of different diameters: The second stage of this study compared spectra from spheroids of different sizes. Small spheroids ($\sim 100\text{ }\mu\text{m}$) were removed on the tenth day post-plating into petri dishes. Note that this is two days later than the small spheroids discussed above because of spectrometer time constraints. The pH was maintained in the range 7.0 - 7.2 for the duration of spheroid growth (35 days).

Spheroid growth curve: For the spheroid growth curves, ~ 50 spheroids were removed and sized using a calibrated reticule in a microscope. For MR studies, spheroids of diameters 150, 250, 400 and 600 and 800 μm were desired. Using the times estimated from the growth curves, spheroids of approximately these diameters were selected by passing the spheroids through a pair of polyethylene filters with grid sizes of 50 μm greater or less than the desired spheroid diameters [100-200, 200-300, 350-450, 550-650 and 750-850 μm] (104). Spheroids of the desired diameter were rinsed off the second filter with fresh medium and placed in the MR tube.

Histological measurements of necrosis: From the population of sized spheroids, ~ 30 spheroids were removed, fixed in 10% formalin, embedded in paraffin and sectioned and stained with eosin and haematoxylin for histological examination. Sections, 5 μm thick, were made throughout the spheroids. Sequential sections were examined under the microscope and sections with the largest diameter of a particular spheroid were used to estimate the percentage of necrosis. This process was repeated for each spheroid. Spheroid shrinkage

following fixation was not more than 10% from initial studies. No correction was made for this. The necrotic volume was estimated as

$$V_{nec} = \frac{4\pi}{3} (r_1 \cdot r_2)^2 \quad 4.1$$

(105) where r_1 and r_2 are the radii of the necrotic core measured under the microscope at right angles to each other. The necrotic fraction of the spheroid was estimated to be V_{nec} / V_{tot} where V_{tot} was calculated in a similar manner to V_{nec} .

Whole spheroids were inserted into a 5 mm NMR tube containing 5mM p-amino benzoic acid as a standard for MR studies. To determine cell numbers, cell size distributions and cell viability, spheroids were disaggregated into suspensions of single cells by firstly aspirating off the medium, then rinsing them twice with citric saline and gently agitating them in trypsin (0.2% w/v in citrate saline) for 10 minutes. An aliquot of single cells was removed and sized using the Elzone Particle Data Counter. Cell viability was assessed by estimating the percentage of cells from disaggregated spheroids which excluded trypan blue dye.

Between 10^7 - 10^8 cells were used for these MR studies. To attain this cell number between 10^2 and 10^5 spheroids (depending on the diameter of the spheroid) were inserted into NMR tubes. However, the volumes of the cells are different in the different cell cycle phases and the cell fragments from necrotic areas were not counted. Thus, spectra are not presented on an absolute scale. Spectra are normalized to the area between 0 ppm and 3.7 ppm enabling this work to be directly comparable to the biopsy studies in Chapter 5. Peak heights and peak height ratios (30,31,92) are presented in the Tables 4.2 and 4.4.

The cell cycle distribution of the cells used in these studies was obtained using standard flow cytometry techniques on a Becton-Dickinson FACscan flow cytometer. Briefly, 10^6 - 10^7 cells were rinsed with ice cold Hanks Buffered Citric Saline, pelleted and resuspended in 70% ethanol and stored at -20°C until flow cytometry was performed. These cells were then sedimented, (1500 rpm) for 5 minutes, resuspended in 1 ml PBS. RNA staining was avoided by adding 100 μl of 1mg/ml RNAase for 1/2 hour. The cell suspension was then treated with 100 μl of 1000 $\mu\text{g}/\text{ml}$ propidium iodide. The frequency of cells in each part of the cell cycle was determined using the fitting software. Details on flow cytometric techniques can be found in Dolbeare *et al.* (106).

4.1.1 MR Methods

^1H MR spectra of the plateau phase and exponentially growing monolayer cells (1D and 2D) and small ($<100\mu\text{m}$) spheroids (1D only because of time constraints due to equipment availability) were measured on an AMX600 (600 MHz) narrow bore Bruker spectrometer. The experimental conditions were the same as those used in Section 3.1.1

MR spectra were measured on spheroids of each desired radius. These measurements were performed on a AM360 Bruker wide bore spectrometer. The MR methods used are discussed in Section 3.1.1. The different sizes of spheroids were not examined using 2D methods again because of equipment availability.

4.1.2 Statistics

Three independent cell preparations for monolayer cultures and spheroids were studied. All results (MR, flow cytometry, cell density, cell size, pH, viability) are presented as average $\pm$ one standard deviation. Regression analysis was done using Microsoft Excel.

4.2 Results

4.2.0 Comparison of Cells Grown To Different Densities

Growing conditions (pH, cell size, cell density and cell cycle distribution) of the cells used for MR studies are shown in Table 4.1. Cells were harvested in early exponential and in plateau phase. The expected decrease in pH of the medium, average cell diameter and percentage of S phase cells is observed as the cell density increases. The growing conditions for small spheroids are also shown.

MR spectra of A2780 ovarian cancer cells in early exponential phase, plateau phase and from 100 μ m spheroids are shown in Figure 4.3. When these spectra are normalized to the area between 0 and 3.7 ppm, the peak at 1.3 ppm is $\sim$ 1.4-fold more intense in the confluent cell preparation than in the exponentially growing cells grown in flasks. The ratio of the peaks at 1.3 ppm to 0.9 ppm has been used as an indicator of mobility (or MR visibility) of triglycerides (13,92). Table 4.2 shows this ratio measured from spectra from exponentially growing cells, plateau phase cells and small spheroids. The intensities of the resonances at 2.09 and 2.34 ppm assigned from the COSY spectra to glutamate are greater in the exponentially growing cells as is the

total choline peak at 3.23 ppm. These resonances are indicated by dashes in Figure 4.3 and their intensities are also presented in Table 4.2.

Table 4.1 Cell cycle phase, size and pH of A2780 ovarian cancer cells growing in monolayers or as small spheroids.

Cells	pH [§]	%S	%G ₁ -G ₀	%G ₂ -M
Early exponential	7.10 ± 0.02	37.6 ± 0.4	49.2 ± 4.0	13.3 ± 4.2
Confluent	6.73 ± 0.02	7.6 ± 1.3	85.7 ± 3.4	6.8 ± 4.7
100 µm spheroids	7.13 ± 0.03	28.5 ± 3.1	51.7 ± 2.1	19.9 ± 1.0
Cells	Cell Diameter (µm) [†]		Cell Density (cells/cm ²)	
Early exponential	14.7 ± 1.7		4.5 × 10 ⁴ ± 0.7 × 10 ⁴	
Confluent	12.7 ± 1.3		8.1 × 10 ⁵ ± 0.4 × 10 ⁵	
100 µm spheroids	14.7 ± 1.4		tissue-like	

† standard deviation of the population of cells

§ pH measured immediately before harvesting

Figure 4.3 ^1H MR spectra (37°C, 600MHz) of the ovarian cancer cell line A2780 growing in a) plateau phase monolayer culture, b) early exponential monolayer culture and c) exponential spheroids.

Table 4.2 Peak ratios from ^1H MR spectra of A2780 ovarian cancer cells at different growth phases

Peak or Peak Ratio [†]	Exponential	Confluent	Spheroid
1.3 ppm / 0.9ppm	2.75 ± 0.21	3.26 ± 0.09	2.65 ± 0.07
2.34 ppm	0.76 ± 0.25	0.22 ± 0.10	0.53 ± 0.05
3.21 ppm	1.67 ± 0.17	0.56 ± 0.06	1.61 ± 0.17

[†] Peak amplitudes or peak amplitude ratios measured at these chemical shifts.

Data are presented as mean ± SD. The two-tailed t-test was used to identify pairs of means that were significantly different ($P < 0.05$). The pairs tested were monolayer exponential vs. confluent and monolayer exponential vs. spheroid. Shaded areas indicate that the means of the pair are significantly different.

No significant differences are seen between cells growing exponentially in monolayers or in spheroids (see Table 4.2).

Representative COSY spectra from plateau phase and exponentially growing cells (from flasks) are shown in Figure 4.4. Note: 2D measurements were not done on spheroids. From these data, cross peak volumes of selected metabolites (Chol, PCho, GPC, glutamate and glutamine) were measured (Table 4.3). These volumes are presented relative to the cross peak arising from the $-\text{CH}_2-\text{CH}_2-$ coupling of lysine at 1.7, 3.0 ppm (92,107). No PEth was observed in any of the cell preparations.

(a)

(b)

Figure 4.4 Representative ^1H COSY spectra from a) plateau phase and b) exponentially growing A2780 ovarian cancer cells.

Table 4.3 Cross peak volume integrals of the selected metabolites of A2780 ovarian cancer cells at different growth phases.

Metabolite [†]	Exponential	Confluent
Choline (Chol)	0.20 ± 0.03	0.30 ± 0.01
Phosphorylcholine PCho	1.10 ± 0.21	0.21 ± 0.05
Glycerophosphorylcholine GPC	not observed	0.09 ± 0.02
Glutamate (Glu)	3.16 ± 0.24	0.79 ± 0.06
Glutamine (Gln)	0.56 ± 0.06	0.51 ± 0.02

† Relative to the cross peak volume of lysine. Data are presented as mean ± SD. Shaded areas indicate that the means of the pair are significantly different using the two-sided t-test ($P < 0.05$).

At the higher cell density, statistically significant decreases in the cross peaks from PCho and glutamate and increases in the cross peaks from GPC and choline were observed.

4.2.1 Spheroids Grown To Different Sizes

The growth curve of the spheroids is shown in Figure 4.5. The kinetics of spheroid growth and tumour growth *in vivo* are similar (108); in general, both exhibit Gompertzian kinetics. Thus, to estimate accurately the times at which the spheroids were at the desired diameter, the curve volume versus time should be fitted to the equation $V_t = V_0 e^{(G/r)(1-e^{-rt})}$, where V_0 was the initial volume and G and r are constants which characterize initial growth and

Figure 4.5 Spheroid growth curve showing the increase in diameter of A2780 ovarian cancer cell spheroids with time.

retardation (109). However, at long times V_t approaches the asymptote $V_0 e^{G/r}$ and this growth curve does not exhibit the later stage where the growth levels off because experiments were stopped before this stage was reached. Therefore, for a rough estimate of the harvest times, the curve (diameter versus time) was fitted to a second order polynomial. The days at which the spheroids have achieved the desired diameter are indicated by triangles.

Sections through spheroids of diameters 350 and 600 μm are shown in Figure 4.6. The viable rim in the larger spheroid is $\sim 150 \mu\text{m}$. The central core contains necrotic cells.

In Table 4.4 spheroid size, percent necrosis and percent cell viability are shown. The percent necrosis is the volume percent of cells estimated from the histological sections. ^1H MR spectra from the different sizes of spheroid are shown Figure 4.7. The peak height ratios of 1.3 ppm to 0.9 ppm are shown in Table 4.5 and Figure 4.8 as a function of spheroid diameter.

Table 4.4 Biological characteristics of different sizes of spheroids

Time (days)	Diameter (μm)	Viability (%) of disaggregated spheroids	Necrosis (%) (not corrected for shrinkage)
10	119 ± 34	86.6 ± 3.2	0
14	268 ± 51	83.5 ± 0.5	0
21	421 ± 62	64.3 ± 6.7	7.5 ± 0.4
28	613 ± 69	49.6 ± 4.8	18.7 ± 4.7
35	804 ± 91	49.5 ± 4.9	24.0 ± 4.9

Figure 4.6 Sections through spheroids of diameters 350 and 600 μm

Figure 4.7 ^1H MR spectra from spheroids of different diameter:
a) 250 μm . and b) 600 μm .

Figure 4.8 Scatter plot of peak height ratios of peaks in the ^1H MR spectra of A2780 spheroids at 1.3 ppm / 0.9 ppm as a function of diameter

Table 4.5 MR peak ratios from different sizes of spheroids

Filter (μm)	1.3 ppm / 0.9 ppm
100 - 200	3.50 ± 0.46
200 - 300	3.66 ± 0.06
350 - 450	3.96 ± 0.13
550 - 650	4.28 ± 0.47
750 - 850	4.80 ± 0.15

Note that the ratio (1.3 / 0.9 ppm) of the small spheroids ($\sim 100 \mu\text{m}$) is not the same as that seen in Table 4.2. There are two reasons for this. Firstly these spheroids were harvested on a different day; flow cytometry was performed on the spheroids harvested on day 10 [%S = 14.5, %G1 79.0, %G2 = 6.5] and thus the proportion of cells in the various phases of the growth cycle are between that observed for exponential and plateau phase cells grown in monolayers. Secondly, these spheroids were measured at a different resonance frequency because of equipment availability. Flow cytometry was not performed on the larger spheroids.

Although the predominant contribution to the 1.3 ppm peak is from the methylene protons from triglycerides (see Figure 4.4), lactate and threonine also resonate at 1.3 ppm. An increase in the ratio of peaks at 1.3 ppm / 0.9 ppm may also indicate an accumulation of lactate within the spheroids (110). However, lactate also resonates at 4.12 ppm. No significant increase in the resonance at this chemical shift was observed.

The peak height ratio $\text{CH}_2 / \text{CH}_3$ correlated strongly with the diameter of the spheroids (regression coefficient = 0.87, Figure 4.8).

4.3 Discussion

Cell metabolism and the external milieu are intertwined. For cell growth, cells use O₂, glucose and growth factors and release lactate, thereby lowering the external pH. It is not fully understood how lowering the extracellular pH affects cell metabolism but it is likely to influence the intracellular pH and cell membranes (111). These factors in turn modulate the response of cells to treatment. For example, the recovery of cells from potentially lethal damage following irradiation is pH dependent (112). Interpreting these effects is difficult because of the complexity of the pathways involved in membrane synthesis.

To help clarify what factors (pH, cell cycle distribution, cell size) predominantly affect the observed spectra, some authors (113) have correlated MR observations with these factors to obtain correlation coefficients. These observations were made on extracted samples. Caution must be made in transferring these techniques to our study because cell volumes and MR visibilities of metabolites are changing and the interactions between the observed metabolites may not depend linearly on the changing biological parameters. The much simpler question asked here is whether the observed MR spectrum from cells at different stages of growth (under standard culture conditions) changes significantly.

A statistically significant increase in the ratio of amplitudes at 1.3 ppm / 0.9 ppm was observed in confluent cells compared to exponentially growing cells. This is in agreement with a recent observation by others (92) who observed a two-fold increase in this ratio as a murine cell line progressed from exponential to plateau phase. With the ovarian cancer cell line used in this

study, only a 20% increase in this ratio was observed. As will be seen in the following chapter, a large range in peak amplitudes in spectra from ovarian tissue and ovarian cancer are observed. It is likely that some of this variation arises from the difference in proliferation status of the cells in the samples. In spite of these variations in the peak heights, these samples provide valuable diagnostic information. However, rather than rely on a limited number of peaks that may be sensitive to the proliferation status (including total choline, the triglyceride peak at 1.3 ppm, and glutamate) the use of the entire spectrum for diagnostic purposes is indicated.

Decreased PCho and increased GPC were observed as cells progressed from exponential growth to plateau phase. These results are supported by a study by Kuesel *et al.* (111) who, using ^{31}P MRS, showed that the level of PCho declined and GPC increased with decreasing pH. Smith *et al.* (113) found that the percentage of cells in S phase correlated with PCho and that there was an inverse correlation with GPC. PEth was not observed in this study but levels of this metabolite depend strongly on the presence of it in the culture medium (111, 114). Ethanolamine was not in the culture medium used but trace amounts would be present in the serum added to the medium.

Elevated levels of glutamate were observed in the exponentially growing cells which probably reflects changes in the energy metabolism of these cells resulting, in part, from changes in the medium concentration of glucose and glutamine (115). Levels of glutamate and glutamine were increased in proliferating lymphocytes (76) and increased glutamine was observed in exponentially growing murine L cells (92).

Spheroids have been used as *in vitro* models of solid tumours for over 25 years (109). Cells in spheroids are grown with cell-cell contact and are held together by microprojections from the surface, the extracellular matrix and

cell-cell junctions (91). Cell shape is also altered when cells are grown as spheroids. In addition, spheroids are not subjected to harvesting procedures (trypsinization or scraping) that may potentially disrupt the membranes. However, the spectral profiles from cells growing as small spheroids were not significantly different from exponential cells growing in monolayers. Thus extending the model of ovarian cancer from single cells to cell aggregates (to take into account cell-cell contact and to reduce sample manipulations) did not significantly change the observed spectra.

The source of the mobile lipid signals in tumours is unknown. They may arise from cancer cells, immune cells or connective tissue (10). Necrotic tissue from Ehrlich carcinomata in mice (116) contained high concentrations of lipid droplets.

The smallest spheroids ($<100\ \mu\text{m}$) had the same growth characteristics and conditions as the monolayer exponential cells whereas the slightly larger spheroids, harvested two days later, had a cell cycle distribution between that of exponential and plateau phase cultures, indicating that the cell population was becoming mixed exponential / plateau phase. The methylene / methyl resonance ratio (1.3 ppm / 0.9 ppm), which has been used to indicate the presence of mobile lipid (92), correlated strongly with spheroid diameter. This, in part, may be due to the change in spheroids from exponential to mixed exponential / plateau phase cells since our monolayer cultures suggest that changes can be observed between cells that are proliferating and those that are in plateau phase.

However, our exponential and plateau phase monolayer cultures were harvested with different media pH values. Kuesel *et al.* (111) showed that levels of membrane associated metabolites depended, not on the pH history of the cells (i.e. the pH of the medium), but rather on the medium pH at the time

of harvest. To ensure that pH artifacts arising from the external medium conditions were reduced, all spheroids were harvested within a small range of external medium pH.

The ratio (1.3 ppm / 0.9 ppm) increased by 20% as monolayer cells moved from exponential to plateau phase. Yet, this ratio increased 40% as spheroids grew from 100 to 800 μm . Thus, cell cycle and pH effects cannot account for all of this change. The presence of a necrotic core (containing membrane fragments) may account for the remaining increase (Tables 4.4, 4.5). This would be in agreement with the brain cancer biopsy samples (10) where it was suggested that necrotic tissue may contain lipid droplets.

4.4 Conclusions

Proliferating ovarian cancer cells exhibit attenuated high resolution ^1H MR lipid signals compared to cells in plateau phase culture. These growing cells also have amplified phosphorylcholine and glutamate signals. Spectra from small spheroids (whole or disaggregated) were not significantly different from monolayer proliferating cells. Thus, cell-cell contact and cell harvesting were factors which did not strongly influence the observed spectra.

An increase in the amount of MR-visible lipid was observed with increasing spheroid diameter. Only part of this change could be accounted for by the changing cell cycle distribution. The remaining increase could be explained by the increasing necrotic fraction in agreement with the large increase in mobile lipid observed in brain cancer biopsies.

Lipids, glutamate and choline resonances will be shown in the next chapter to have diagnostic significance. We have shown that these resonances are sensitive to growth conditions.

CHAPTER 5   HUMAN OVARIAN CANCER (*EX VIVO*)

In this study we evaluate whether employing multivariate techniques, in particular linear discriminant analysis (LDA), can improve the sensitivity and specificity in the classification of ovarian neoplasms and further, whether within the subgroup of cancer biopsies, recurrent cancers can be distinguished from untreated tumours.

5.0   Introduction

Proton spectra of biological specimens are inherently complex because of the large number of hydrogen-containing metabolites. Consequently, ^1H spectra contain a large amount of biological information, of which only a fraction may be important for distinguishing between different classes of samples. For example, to classify cancer samples from normal tissue, it is necessary to identify those spectral regions important for discrimination. Variations in these spectral regions arise not only from biochemical differences between the two classes but also from sample heterogeneities within a given class.

In the previous chapters we have established that there are significant differences between cells which are sensitive or resistant to chemotherapy. We have also shown that ^1H MR spectra from cells grown in different environments (monolayer exponential, monolayer plateau, spheroids of increasing size) have markedly different MR profiles. In biopsy samples we expect that, in addition to the different microenvironments found within tumours and the different drug sensitivities of cancer cells, there will be contributions to the MR spectra from immune cells, fat and vasculature. To

complicate matters further, there are many histological types of ovarian cancers as shown in the introduction (Table 1.1).

Despite the inherent complexity of tumour biopsies, a recently published study (31) has shown that ^1H MRS can successfully distinguish between normal/benign and malignant human ovarian cancer, with a sensitivity of 87% and a specificity of 91%. The discrimination between these two groups was based on the peak height ratios of resonances at 3.0 ppm (creatine, phosphocreatine, lysine) to 1.7 ppm (lysine, polyamines) and 1.7 ppm to 1.3 ppm (lipid, lactate, fucose, threonine).

Thus, three spectral regions were identified for discrimination. However, there may be other spectral regions which may improve the discrimination between the classes. Additional biochemical differences between classes can also be used to further our understanding of the mechanisms which lead to the malignant transformation of cells. Spectral areas of potential significance can be obtained using multivariate techniques.

Statistical pattern recognition techniques were used to classify ^1H spectra from perchloric acid extracts of normal rat tissues and rat tumours (52). These samples could not be distinguished on the basis of any single metabolite. By firstly preprocessing the data using principal component analysis, then classifying with cluster analysis, almost all the different tissues and tumours could be distinguished. However, in their study, tumours were not compared to the normal tissue types from which the tumours arose. This and the fact that samples were extracts of rat tissue reduces the relevance of their study to human studies.

Excellent discrimination between different classes of human thyroid cancer has been achieved using multivariate techniques (11). These data were either preprocessed using principal component analysis or simply by obtaining peak

amplitudes. Comparable results were obtained whether classification was performed on the peak amplitudes or on the set of principal components which accounted for 98% of the variance. Several classification techniques, singly or in combination, have proved successful in distinguishing between ^1H spectra obtained from different classes of samples. A comparison of several techniques (neural nets, cluster analysis, genetic programming, LDA) classifying the same data set can be found in the study by Somorjai *et al.* (11). No single classifier outperformed the others and thus the simplest and most robust of the techniques, LDA, was chosen to analyse our ovarian cancer biopsy spectra. LDA was performed on spectral amplitudes rather than on the set of principal components. The advantage of using peak amplitudes is that the biochemical properties of the different classes can more readily be retrieved.

Employing preprocessing (data reduction) techniques developed in-house (53), and LDA, allow the immediate identification of the spectral regions which are important for classification. Such techniques will lead to improved classification of normal ovaries and ovarian neoplasms. In addition, by using multivariate methods, recurrent tumours (from patients who have undergone unsuccessful chemotherapy) may be distinguishable from untreated ovarian cancer. Spectral regions that distinguish between treated and untreated ovarian cancer will be compared to those regions which distinguish the resistant ovarian cancer cell lines from their sensitive parents (Chapter 3). This key part of the study is an important step toward assessing whether ^1H MRS can detect chemotherapy-induced changes in *ex vivo* ovarian cancer and further, whether these regions are indicative of drug-resistance.

5.1 Materials and Methods

5.1.0 Ovarian Biopsy Samples (Normal, Benign, Borderline and Malignant)

Biopsy samples of postmenopausal ovaries, benign ovarian neoplasms and ovarian cancer (~0.5 g) were removed during surgery at the Ottawa General Hospital by Dr. M. Fung Kee Fung. They were placed in cryogenic vials containing PBS/D₂O and were immediately frozen. The histological types of these samples were normal (19), benign (3), borderline (3), serous (13), endometrioid (17), mixed epithelial (3), small cell (4) and malignant teratoma (2). For MRS studies, samples were thawed and a small section of the biopsy sample was removed (0.03 - 0.07 g). Care was taken to exclude obvious fatty areas and vasculature. These samples were washed three times with PBS/D₂O and placed into capillaries (ID 3 mm) containing PBS/D₂O and were stoppered with a teflon plug (see Figure 5.1). The capillary was inserted into a 5mm NMR tube containing a 5mM solution of p-amino benzoic acid as a reference. This procedure is detailed in Kuesel *et al.* (12). Following MR studies, the sample was fixed in a solution of 10% buffered formalin for histological examination.

The diagnosis and evaluation supplied by the pathologist included, in addition to the histological type and grade, the degree of necrosis (high, medium, low), whether the tumour was a primary or secondary (only primaries were included) and whether the patient had undergone chemotherapy. Histopathological diagnosis was performed by Dr. M. Senteman at the Ottawa General Hospital.

To assess intra-tumour heterogeneity, duplicates (3-4) of five cancer samples were measured.

Figure 5.1 Biopsy sample (0.3-0.7 g) in a capillary containing PBS/D₂O buffer. The capillary is stoppered with a teflon plug and placed in a 5mm NMR tube containing 5mM PABA in PBS/D₂O.

5.1.1 MR Methods

Acquisition

1D and 2D COSY ^1H spectra were collected with the same acquisition parameters used in Chapter 3. Following the measurement of the 1D and 2D spectra a second 1D spectrum was obtained to ensure that, during the course of the MR measurements, the sample had not degraded sufficiently to compromise the MR measurements.

Processing

For measuring peak amplitudes, 1D spectra were processed in the manner discussed in Chapter 3. For LDA analyses, the 1D spectra were processed using software written in-house (at the Institute for Biodiagnostics, National Research Council). Spectra were Fourier transformed without applying line broadening. Zeroth and first order phase corrections were applied using an objective phasing routine developed in-house, which maximized the positive signal intensity. These spectra were normalized to the area between 0 ppm and 3.7 ppm. Reduction of the number of variables in the spectra (i.e the number of frequency versus intensity points) was accomplished by averaging the intensity over five adjacent frequency points. Only the averaged intensities between the chemical shift range 0 and 4 ppm were analysed using the classifiers; thus, the total number of attributes (or averaged intensities) was 110 for each spectrum.

These spectra were then assigned randomly into a training set and a test set of data, ensuring that each set contained roughly the same proportion of

normal to cancer samples. The training set was then analysed using a program developed in-house (53). This program (Optimal Region Selector) searched for the best discriminating vector for all combinations of attributes. In practice, ten or fewer attributes were sufficient. The attribute selection criterion is the simultaneous minimization of classification error and classification ambiguity ("fuzziness"). LDA, with leave-one-out (LOO) cross validation to render it more robust, was used. The minimum number of attributes which accurately classified the training set was selected and the best discriminating vector for this set of attributes was used to classify the test set. Thus, having trained the LDA classifier to distinguish between two classes (for example between normal and cancer), the members of the test set were assigned to one of these classes. The LOO method was used at this stage to assign a degree of membership of a sample to a given class (see Chapter 2). This gives an estimate of the "fuzziness" of the class assignment. Samples which can not be reliably classified, i.e. samples whose probability of belonging to one of the classes is <75%, can, if desired, be examined using other analysis techniques (11). All analyses were performed on a Silicon Graphics Instrument (SGI Challenge Series Server).

COSY FIDs were processed using a sinebell weighting function over 512W in t_2 (using the conventions described in the theory section) and 160W in t_1 , zero filled in t_1 to 1K and Fourier transformed in both dimensions. As before (Chapter 3), COSY spectra are presented in magnitude mode and were used, in part, to assign resonances in the 1D spectra. In addition, resonances were assigned using literature values (75,76) and with the addition of standard substances.

5.1.2 Training Sets and Test Sets

For the classification between normal and cancer (treated and untreated) the training set comprised 12 normal and 22 cancer and the test set 7 normal and 15 cancer. In addition, a second test set containing benign and borderline neoplasms and duplicate samples from the same patient (spectra measured to assess tumour heterogeneity) were classified using the original training set containing normal ovaries and malignant ovarian cancer. All duplicates were classified into the same group as the representative sample chosen for the test set. These data are not presented. Classification was repeated on another set of training and test sets which contained data that were preprocessed using standard Bruker software. These sets gave exactly the same results as the spectra processed using software developed in-house and so the data will not be presented. To determine whether untreated and treated cancers could be distinguished, LDA was performed on the subset of malignant cancers (excluding the malignant teratomas) [10 treated and 25 untreated]. This set was not divided into a test set and a training set. LDA was done with LOO, and region selection identified 10 discriminating regions out of 110.

5.2 Results

5.2.0 Biopsy Samples (Histology and MR Spectra)

Histological Sections of Biopsy Samples

Representative histological sections of fixed normal, benign, borderline and cancer biopsies are shown in Figure 5.2. These samples were stained with haematoxylin and eosin and are sectioned 2-3 μm thick. Microscopically, the architectural patterns of the benign neoplasm resemble that of the normal

Figure 5.2a,b Histological sections of a) normal postmenopausal ovary and b) benign neoplasm stained with haematoxylin and eosin (magnification 200X)

Figure 5.2c,d Histological sections of c) borderline and d) frankly malignant ovarian cancer samples stained with haematoxylin and eosin (magnification 200X).

tissue whereas malignant neoplasms exhibit a reduced level of normal architecture and reduced cellular differentiation (i.e., cells no longer resemble the normal cells of the tissue from which they arose). Nuclei from benign neoplasms are also of normal size. By contrast, nuclei from malignant cells are enlarged, have irregular shape and exhibit an increased number of mitoses.

Typical Spectra with Assignments

Representative 1D ^1H spectra from normal, benign, borderline and cancer biopsy samples removed during surgery are shown in Figure 5.3. These spectra are normalized to the area between 0 and 3.7 ppm, but have not had their dimensionality reduced by averaging the intensity over five frequency points. In agreement with the findings in Chapter 3, the spectra are dominated by resonances from lipids [methylene (1.3 ppm) and methyl (0.9 ppm) groups], cholines (3.2 ppm), creatines (3.0 ppm) and lysine and polyamines (1.7 ppm). The spectra from the benign neoplasms resemble normal spectra. Both have less intense lipid signals than the borderline serous and frankly malignant tumours. In contrast to the results of Mackinnon *et al.* (31), the borderline serous profile more closely resembles its malignant counterpart than the benign and normal spectra.

2D COSY spectra of normal ovaries and ovarian cancer are shown in Figure 5.4. These spectra present crosspeaks from methyl and methylene moieties on fatty acyl chains, amino acids, lactate and cell surface fucose. In agreement with the study of MacKinnon *et al.* (31), spectra from the normal ovaries show no presence of cell surface fucosylation whereas the frankly malignant tumours exhibit multiple resonances from cell surface bound fucose at (4.13 ppm:1.3 ppm).

Figure 5.3 ^1H MRS spectra from normal, benign, borderline and frankly malignant ovarian cancer samples.

a)

b)

Figure 5.4 2D COSY spectra measured at 360 MHz from a) normal ovaries and b) ovarian cancer

Comparison of spectra from five histological types of ovarian cancer are shown in Figure 5.5. In agreement with the study by Mackinnon et al. (31), spectra from malignant endometroid (grade III), serous (grade III) and mixed epithelial (grade III) ovarian cancer are similar. By comparison, the two teratomas studied exhibited broad signals. Teratomas are heterogeneous and can contain many cell types, hair and teeth. They were not included in the multivariate studies. Small cell ovarian cancers are not graded, they all behave aggressively. The peaks are narrower compared to the other histological types.

Intra- Tumour Heterogeneity

Spectra from three endometroid tumours are shown in Figures 5.6a-c. In Figure 5.6a the range and average of intensities of spectra obtained from an endometroid tumour which contained areas of necrosis are shown (n.b. no gross necrotic areas were visible during sample preparation). Spectral intensity ranges and averages of two additional endometroid tumours (one with no necrosis and one with a small amount of necrosis from a recurrent tumour) are shown in Figures 5.6b,c. Tumours with regions or micro regions of necrosis exhibited significant variability in the intensities of the lipid regions.

5.2.1 Classification

Classification of Normal versus Cancer (Amplitude Ratios)

The ^1H MR spectra from normal ovaries, benign growths, borderline and malignant ovarian cancer were initially analysed using the peak amplitude ratio

Figure 5.5 Comparison of the ^1H MRS spectra of five histological types of ovarian cancer; serous, endometrioid, mixed epithelial, small cell and malignant teratoma.

Figure 5.6 Mean and range of intensities observed in ^1H MRS spectra from three to five sections from each of three endometroid ovarian cancers a) necrotic, b) non-necrotic and c) very little necrosis, recurrent tumour

Figure 5.7 Scatter plot of peak amplitude ratios (3.0 ppm / 1.7 ppm) vs (1.7 ppm / 1.3 ppm) obtained from the ^1H MRS spectra from normal, benign, borderline and frankly malignant ovarian cancers. The solid line indicated the line distinguishing normal and benign tissue from malignant ovarian cancer. The dashed line indicates the slope of the equivalent line presented by Mackinnon *et al.* (31).

method of Mackinnon *et al.* (31). A scatter plot of the peak amplitude ratios (1.7 ppm / 1.3 ppm) and (3.0 ppm / 1.7 ppm) is shown in Figure 5.7. Normal samples could be distinguished from malignant samples with a sensitivity of 0.95 and a specificity of 0.89. The slope of the line separating these two classes was different from that found by Mackinnon *et al.* (31). The amplitude ratio method is the graphical equivalent to a one-shot resubstitution approach (i.e. no cross validation) with no training versus test set.

In light of the finding by Kuesel *et al.* (10) that necrotic samples had elevated lipids and the observation above that significant intra-tumour spectral variations are present in necrotic samples, the peak amplitude ratio (1.7 ppm / 1.3 ppm) was calculated for tumours which contained areas of necrosis and for tumours which did not. These ratios are given in Table 5.1.

Table 5.1 Ratio of amplitudes at 1.7 ppm to 1.3 ppm from necrotic and non necrotic ovarian cancer biopsy samples.

Peak Ratio	Necrotic†	Non Necrotic	2-tailed p value
1.7 ppm / 1.3 ppm	0.19 ± 0.11	0.42 ± 0.09	<0.0001

† mean plus or minus standard deviation

Classification of Normal Versus Cancer (LDA)

To establish the range of intensities that are present in two classes of the training set of biopsy samples (normal and cancer), the mean and range of intensity values of the normal and cancer populations are shown in Figure 5.8. These spectra have their dimensionality reduced (i.e. have undergone data

Figure 5.8 Range of intensities of data-compressed normal postmenopausal ovaries (a) and ovarian cancer (b) in the training set. Spectra within the training set were normalized to the area between 0.0 and 3.7 ppm and are presented on the same vertical scale. Standard deviations of each averaged intensity at a given chemical shift were not found to be statistically significant

compression) as discussed in the methods section and thus the resolution is reduced. The means of the two populations are replotted in Figure 5.9 to allow the direct comparison of the two populations. Using the unpaired t test, the differences between each class-averaged attribute (i.e. the means and standard deviations of each of the 110 intensities) were found not to be statistically significant.

For this study, the minimum number of attributes which gave 100% discrimination between classes in the training set was chosen. Six attributes were required to classify the training set with an accuracy of 100%. A typical output table from LDA classification of MR spectra is given in Appendix 6. In this table it can be seen that certain combinations of attributes give the best discrimination between classes and as some additional attributes are incorporated into the classification, the discrimination power increases. The chemical shifts and the major metabolites contributing to that spectral region are presented in Table 5.2. These attributes are indicated in Figure 5.9 with vertical lines.

Using these six attributes, LDA was able to classify the test set with high accuracy (95%). Classification results are summarized in Table 5.3. LDA was able to classify normal and cancer samples with a sensitivity of 1.00 and a specificity of 0.95 and an overall accuracy of 98% (averaged over the training and test samples). The assignment of the additional test set containing the benign and borderline samples is also given in Table 5.3.

Figure 5.9 Data-compressed class-averaged ^1H MRS spectra from normal ovaries and ovarian cancer in the training set. The vertical lines indicate the attributes used to classify the training set.

Table 5.2 Attributes identified by LDA for distinguishing between normal ovaries and ovarian cancer and untreated and recurrent ovarian cancer

Chemical Shift (ppm) [†] Cancer vs Normal	Chemical Shift (ppm) [†] Untreated vs Recurrent	Attribute
-	0.82	-CH ₃
-	0.92	-CH ₃
-	1.32	-(CH ₂) _n
1.47	-	-OOC-CH ₂ -CH ₂ -
1.68	-	lysine
-	1.92	-CH ₂ -CH ₂ -CH=
-	2.33	glutamate
2.80	2.76	=CH-CH ₂ -CH=
2.97	-	total creatine
3.17	3.20	total choline
3.34	3.30	taurine
-	3.63	PCho
		GPC
-	3.70	glu / gln / GSH

† because of data compression, the errors on the chemical shifts are ± 0.02 ppm

Protons contributing to the assigned resonance are presented in bold face.

Table 5.3 Classification of normal ovaries and ovarian cancer

<u>Class</u>	<u>Classified Normal</u>	<u>Classified Cancer</u>	<u>% Correctly Assigned</u>
<u>Training Set</u>			
Normal (n=12)	12	0	100.0
Cancer (n=22)	0	22	100.0
<u>Test Set</u>			
Normal (n=7)	6	1	85.7
Cancer (n=15)	0	15	100.0
<u>Test Set 2</u>			
Benign (n=3)	1	2	
Borderline (n=3)	0	3	

Classification of Recurrent and Untreated Ovarian Cancer (LDA)

Histological sections from untreated and recurrent moderately differentiated serous ovarian cancer are shown in Figure 5.10. No significant morphological differences are evident between untreated and treated ovarian cancer. Their class averaged spectra are shown in Figure 5.11 with the ten attributes used for classification shown by vertical lines. Again, using the unpaired t-test, the differences between the class-averaged attributes were not found to be statistically significant. The chemical shifts and metabolite assignments are also given in Table 5.2. LDA was able to distinguish between untreated and recurrent ovarian cancer with an overall accuracy of 97% (see Table 5.4). Removal of the single fuzzy sample, i.e. the sample whose probability of belonging to one of the classes is <75%, increased the accuracy to 100%.

Figure 5.10 Histological sections from a) untreated and b) recurrent moderately differentiated serous ovarian cancer stained with oesin and haematoxylin (magnification 200X)

Figure 5.11 Data-compressed class-averaged ^1H MRS spectra from untreated and recurrent ovarian cancer. The ten attributes used for classification are indicated by vertical lines.

Table 5.4 Classification of untreated and recurrent ovarian cancers

<u>Class</u>	<u>Classified Untreated</u>	<u>Classified Recurrent</u>	<u>% Correctly Assigned</u>
<u>Training Set</u>			
Untreated (n=25)	25	0	100.0
Recurrent (n=12)	1*	11	91.7

* identified as a "fuzzy" sample.

5.3 Discussion

The differences between normal tissues and cancers originating from those tissues vary from one organ to another. ^1H spectra from normal colorectal tissue are characterized by broad lipid peaks. Malignant colorectal tissue can be distinguished by the narrowing of the linewidths of the triglyceride methylene resonances, increases in the levels of choline based metabolites and increases in cell surface fucosylation (32). Normal cervix and preinvasive cervical cancer do not have a pronounced lipid spectrum whereas the invasive cancer does. Spectral regions used for distinguishing cancer from preinvasive cancer are 4.15-3.5 ppm and the choline and creatine resonances at 3.2 and 3.0 ppm respectively (12,13). Brain tissue is characterized by the absence of a lipid spectrum (10,30). Glioblastomas and metastases can be distinguished from normal brain tissue on the basis of the spectral regions from 3.4-3.1 ppm and 1.5-1.1 ppm (9).

Thus, many studies have compared cancerous and preinvasive growths to normal tissue. The regions used for discrimination include lipids, choline and

creatine metabolites which were also identified in this study. Using multivariate techniques additional spectral regions can be identified and the relative importance of these regions can then be compared, from one tissue to another, to see whether a biochemical picture emerges as normal tissues progress through a precancerous stage (if it exists) to being malignant.

Using the technique of Mackinnon *et al.* (31), normal post-menopausal ovaries were grouped separately from malignant ovarian cancer. The slope of the line separating these two groups was different in our study. This may possibly be due to differences in the preprocessing of the spectra (baseline correction, phasing), differences in sample preparation or limited sample size in both studies. To reduce any user errors introduced in spectral processing, spectra used for LDA were processed using an objective phasing algorithm developed in-house and no baseline correction. However, these spectra were also processed using standard Bruker software, and the results of the multivariate analysis were identical to those obtained using the objective algorithms. Thus, although hand phasing and using an objective phasing algorithm gave the same results in this study where the phasing was performed by one person, objective preprocessing becomes a more important issue when data from several sources are combined.

The important classification regions identified by Mackinnon *et al.* (31) were the resonances at 1.3 ppm, 1.7 ppm and 3.0 ppm. In our study we identified the regions 3.3 ppm, 3.2 ppm, 3.0 ppm, 2.8 ppm, 1.7 ppm and 1.5 ppm. The spectral region at 1.3 ppm was not identified. However, what should be considered is that the spectra in this study are normalized to the area between 3.7 and 0 ppm, whereas in the Mackinnon study ratios are taken, and the spectra are effectively normalized to the height of the tryglyceride methylene peak at 1.3 ppm. One disadvantage of their approach is that the

height of this peak may be sensitive to the degree of necrosis or grade of the tumour. Determining the importance of this is made difficult by the fact that most (93%) of the higher grade tumours in this study had areas of necrosis (although care was taken to exclude any necrotic areas visible to the eye). This may imply that classification between normal and cancer may be based in part on the presence or absence of necrotic regions. The additional regions found in this study may provide more complete biochemical information.

The samples that were misclassified using the two techniques were different. This observation has recently been exploited by Somorjai *et al.* (11), who have analysed spectra using several independent classifiers, and combined their outcomes to reach a consensus diagnosis. Samples which cannot be confidently classified are identified and can be subjected to additional analyses.

Ovarian cancer is asymptomatic in its early stages, which means that it is very difficult to diagnose until it is well advanced and more difficult to treat. Surgery and subsequent therapy are usually dictated by the degree of spread (117). A potential role of MR may be in the early detection of ovarian cancers in high risk women (i.e. with a family history of the disease). However, to completely characterize ovarian cancer a huge data set is needed which has many (~20) representative samples from each histological type and grade. In addition, all the benign conditions that are present in normal ovaries, including any changes that occur during the menstrual cycle, must be investigated.

Perhaps more importantly, if, in addition to a diagnosis, MR could provide information on the relative resistance to a course of therapy, this would be of enormous benefit, saving the patient from extremely costly and possibly fruitless treatment, and allowing the physician to customize the treatment for each patient.

Using LDA, untreated ovarian neoplasms could be distinguished from recurrent ovarian tumours. One interpretation of this finding is that ^1H MRS can detect chemotherapy-induced changes in *ex vivo* ovarian neoplasms. Some of these metabolite differences may represent cellular changes that occurred during the onset of resistance. Of the regions identified as important for classification, several were identified in our studies on single human ovarian cancer cell lines. These were the regions 0.9 ppm (methyl groups from lipids and proteins), 1.3 ppm (predominantly from the methylene group of triglycerides), 2.34 ppm (glutamate), 3.2 ppm (total choline), 3.3 ppm (taurine). As mentioned, no single metabolite was significantly different in the recurrent tumours compared to the untreated tumours. However, treatment failure arises not only from cellular resistance, which is itself multifactorial (GSH levels, changes in membranes, increased DNA repair capacity) but also from inadequate drug therapy or from differences in patient-drug metabolism. Salvage therapy is not effective in 90% of recurrent tumours because the tumours are either intrinsically resistant or have developed resistance.

We would also expect ~30% of the untreated tumours to be intrinsically resistant. The mechanisms that give rise to "intrinsic" vs. "acquired" resistance are not well characterized and may or may not be the same. With sufficient sample set sizes that are retroactively assigned to being sensitive, intrinsically resistant or having acquired resistance, ^1H MR may be able to identify cellular differences between these classes.

A second interpretation of these findings is that these recurrences can be distinguished from the primary tumours because they arise from metastases which may have grown under different hormonal conditions compared to the primary or from outgrowths of a regionally resistant population in the primary. Further work is needed to evaluate whether differences between recurrent

tumours and ovarian cancer primaries arise from chemotherapy-induced changes or from different tumour growth conditions. With the extremely low response rate of recurrent tumours to second line therapy, the understanding of any post-chemotherapy changes may provide important information about induced resistance *in vivo*.

5.4 Conclusions

LDA improves the accuracy and reliability of the classification of normal ovaries and ovarian neoplasms and is able to distinguish between untreated and recurrent cancers. ^1H MRS shows promise in characterizing the properties of ovarian cancer cells which are resistant to therapy. Future *in vivo* studies of ovaries will benefit from the data and analyses developed in this study.

CHAPTER 6 ^{31}P MRS OF DRUG-SENSITIVE AND DRUG-RESISTANT OVARIAN CANCER CELLS

6.0 Introduction

Although the identification of therapy-resistant cells is useful to predict patient outcome it is, perhaps, more important to determine whether the changes in control of metabolic pathways that occur following the induction of resistance can be overcome using alternative therapies. The observation of cell metabolism by MR spectroscopy prior to and following treatment necessitates that cells be in an environment that can sustain cell viability and growth. Differences between drug -sensitive and -resistant ovarian cancer cells suspended in PBS/D₂O were observed using ^1H MRS (Chapter 3). However, cells in deuterated buffer remain viable for relatively short periods (<4 hours) and are known to metabolize differently than when incubated in growth medium (118). Cell viability can be maintained at 37° for up to 4 days in the magnet by perfusing cells with growth medium (72,102). Unfortunately the large water resonance from the perfusate can interfere with important resonances in the ^1H spectra. Complications associated with water suppression can be avoided by studying the cells by ^{31}P MRS.

^{31}P MRS has proven to be successful for non-invasive monitoring of the metabolism of intact viable cancer cells (72,73,102,119). Studies on cells have concentrated on differences in levels of energy metabolites; phosphocreatine (PCr), nucleosidetriphosphates (NTP), inorganic phosphate (P_i) and phospholipid metabolites; phosphomonoesters (PME) and phosphodiester (PDE). Thus, phosphate metabolites provide a window into changes in phospholipid turnover and energy requirements in cells. An additional

advantage of ^{31}P MRS over ^1H MRS is that fewer metabolites are present in the spectra, making quantification easier.

Increased levels of PCr have been observed in adriamycin-resistant mutants derived from both human breast (72) and lung cancer cell lines (73). In comparison to their parental cell lines, PME and PDE resonances were lower in the mutant breast cancer cell line and unchanged in the resistant lung cancer cell line. Cell lines selected for resistance to adriamycin may exhibit the classic multidrug resistant (MDR) phenotype in which the 170 kD P-glycoprotein is overexpressed. This P-glycoprotein functions as an energy-dependent pump to expel adriamycin from the cell and hence differences in the energy requirements of cells sensitive and resistant to adriamycin are not surprising. Adriamycin is not commonly prescribed for ovarian cancer patients whereas chemotherapy incorporating cisplatin or cisplatin analogues is the preferred method of treating women with advanced disease (16). It is not known whether the differences in mechanisms responsible for cisplatin-resistance are energy-dependent or associated with differences in membrane turnover.

This study investigates whether levels of ^{31}P MRS metabolites in cisplatin-resistant ovarian cancer cells differ from -sensitive cell lines. To compare these results with the studies discussed above, ^{31}P spectra of cell extracts of the adriamycin-resistant cell line, 2780^{ad}, were also measured. This work, combined with the ^1H MRS study in Chapter 3, may provide an initial step towards predicting responses *in vivo*.

Cell Entrapment

To maintain viable cells within the magnet cells must be maintained in a carefully controlled environment. Cellular metabolite levels are sensitive to O₂, temperature, nutrient levels and pH. Secondly, as a consequence of the low sensitivity of ³¹P MRS, large numbers of cells (10⁷ - 10⁸) are needed to achieve an adequate signal-to-noise ratio (SNR). Care must be taken to supply these cells with adequate amounts of O₂ and nutrients and to remove waste products while providing a sterile, 37° environment. A cell perfusion system (72,102) can provide the conditions essential for maintaining viable cells and has the additional advantage that these conditions (for example temperature or drug concentration in the medium) can be rapidly changed. This type of system can therefore be used to evaluate the metabolic response of cells to drugs, hyperthermia and radiation.

When incubating in a perfusion system, cells must be constrained within the NMR receiver coil or would be washed away with the perfusate. Several systems of entrapping cells have been used and will be discussed briefly (120).

Cells can be attached to dextran microcarrier beads (or beads made with other materials). The disadvantages of this technique include a) reduced SNR because of the dead volume associated with the beads, b) poor field homogeneity (probably due to the movement of the beads, c) studies limited to anchorage dependent cells and d) there is a loss of cells through the detachment of dead and dying cells from the surface, particularly following treatment.

Cells can also be grown on or in hollow fibres. The distribution of nutrients is often inadequate and retrieval of cells for survival studies is not easily accomplished. Thus, quantification of spectra with respect to cell

numbers is difficult and observed spectral changes cannot be correlated to changes in the clonogenic potential of the cells.

Another common method is to entrap cells within agarose. With this technique cells do not proliferate and the penetration of very large molecules into the gel has not been determined. Cells cannot easily be retrieved from agarose. Basement membrane gels have also been used to embed cells in threads but are more difficult and expensive to work with; these gels are fragile and cells are easily lost.

Entrapment in alginate beads has the advantage that a) the beads are stable, b) cells can easily be retrieved for cell survival studies and c) they can be used to assess the effects of drug or radiation treatment. For these reasons we chose to entrap cells in alginate beads for this study. Ng *et al.* (102) have used this system to study the response of RIF-1 mouse fibrosarcoma cells to radiation. Although the work in this chapter only compares the steady state phosphorus spectra of untreated cells, this system can be used to assess the response of cells to drugs and radiation (see Chapter 7 for future directions). It is therefore necessary to be able to correlate the MR measurements with the traditional measure of survival, the clonogenic potential of cells determined using the colony forming assay. In the first part of this study, the entrapment of cells in alginate is characterized to determine whether a) cells grow in alginate and b) whether cells retrieved from alginate retain their clonogenicity. The second stage of this study is to compare the ^{31}P MR spectra of a sensitive ovarian cancer cell line with two resistant mutants. Spectra from extracted and intact cells will also be compared.

6.1 Materials and Methods

6.1.0 Cell Methods

In addition to two of the ovarian cancer cell lines studied previously (A2780, 2780^{CP}) an adriamycin resistant mutant was also investigated. This cell line, 2780^{ad}, was kindly provided by Dr. J. Gerlach, Kingston, Ont. Resistance was induced by exposing the parental cell line (A2780) to stepwise increasing concentrations of adriamycin (121). This cell line exhibits the classic pleiotropic phenotype. All three cell lines used in this study (A2780, 2780^{CP} and 2780^{ad}) were grown in the culture conditions described in Chapter 3 and were harvested for MR studies in plateau phase.

Cells were entrapped in calcium alginate beads using the procedure described by Ng *et al.* (102) and Narayan *et al.* (122). Following trypsinization, cells were rinsed twice in Ca^{2+} , Mg^{2+} -free Hank's balanced salt solution and 10^8 cells were resuspended in the same buffer. This mixture was combined with 1.1 ml of 2.4% (w/v) sodium alginate (102,122). The cell mixture was then extruded through a 27 gauge needle with the bevel removed into 100 mM CaCl_2 (see Figure 6.1) to form spherical calcium alginate beads (<2 mm in diameter). The beads were rinsed with medium several times prior to transferring them to the perfusion system (for MR experiments) or spinner flasks (for bead characterization). Both the perfusion system and the spinner flask contained 350 ml of medium.

Growth of cells in beads incubated in spinner flasks for 72 hours was assessed by releasing cells from a known number of beads by exposure (5-10 min.) to sodium citrate (100 mM). Cells were then rinsed with medium and separated into three tubes to a) count the cell number, b) plate the cells for the

Figure 6.1 Preparation of calcium alginate beads. Cells, suspended in buffer, are combined with sodium alginate and extruded through a 27 Gauge needle into 100 mM Calcium Chloride to form calcium alginate beads (<2mm in diameter).

colony forming assay and c) fix the cells in 70% ethanol for cell cycle analysis. These measurements were made on cells prior to encapsulation, immediately following encapsulation and 24, 48 and 72 hours following encapsulation. Beads were also retrieved at these times and fixed in formalin for histological examination.

Standard flow cytometric techniques were used to obtain the cell cycle distribution (see Chapter 4). Cell cycle analysis was performed on the Ortho Cytofluorograf 50H at the Institute for Biological Sciences, National Research Council, Ottawa. Cell counting and the colony forming assay are described in Chapter 3.

6.1.1 Perchloric Acid Extracts

Extracts were treated following the method of Kuesel *et al.* (111). Briefly this involved the following steps. Cells were harvested and cooled to 4° C for the remaining procedures. Cells (10^8) were spun down at 1500 rpm. The resulting pellet was rinsed and spun down twice with 20mM HEPES (pH 7.3) and resuspended in 2 ml of the same buffer. Perchloric acid (2ml of 0.5 M) was added to the cell suspension, and the mixture was kept on ice for 30 min (periodically sonicating), and centrifuged for 5 minutes. The supernatant was neutralized with K_2CO_3 , kept on ice for a further 30 minutes and centrifuged. The neutralized extracts were immediately lyophilized. For MRS studies, the extracts were redissolved in 20mM HEPES, 10mM EDTA, with D_2O added for a spectrometer lock signal. The pH was adjusted to 7.3. A reference capillary containing dimethyl methylphosphonate (DMMP) was inserted as a standard.

6.1.2 Perfusion System

A closed perfusion system was designed to satisfy all the requirements necessary for incubating the cells in a magnet and which could, in addition, be used in a 10mm combined ^{31}P - ^1H probehead. This system is illustrated in Figure 6.2. The alginate beads were placed between the two perforated teflon inserts in a 10mm screw cap NMR tube (Wilmad, Bueno, NJ). Perfusate leaving the reservoir was pumped through a gas permeable silastic tubing lung enclosed in a jacket containing 95% O_2 / 5% CO_2 . The medium was then transferred to polyethylene tubing (PE190) and passed through a heated water jacket (38°). The medium dropped into a bubble chamber and then flowed down the tubing to the bottom of the NMR tube from where it flowed upwards past the beads and was then returned to the reservoir. The flow rate of the perfusate was 6 ml/min and the medium reservoir was kept at 38° to attain a constant medium temperature of 37° flowing past the beads. The system was sterilized with 0.05N NaOH. For the MR experiments penicillin and streptomycin were added to the perfusate.

Cell culture facilities were at the Ottawa Regional Cancer Centre (ORCC) and the MR facilities were at the Institute for Biodiagnostics, National Research Council. Entrapped cells were prepared at the ORCC, inserted into the 10mm NMR tube and transported on ice to NRC. The NMR tube was connected to the perfusion system and cells were perfused for 2 hours prior to MR measurements.

Figure 6.2 Cell perfusion system

6.1.3 MR Methods

³¹P MR Spectra were obtained on a Bruker AM360 Spectrometer operating at 145 MHz. Spectra were collected with a sweep width of ± 5952 Hz, 8K data points, 40° pulse angle and a repetition delay of 2s. For the extracts 5×10^4 free induction decays were collected and, prior to Fourier transforming the spectra, a 2 Hz line broadening was applied. For the perfused intact cells, 1200 transients were acquired and a 20 Hz line broadening was used. The chemical shift was set relative to PCr at 0 ppm (the reference DMMP was set at 41.5 ppm). Extracts were run at 12° C and perfused cells were maintained at 37°

Spectra measured under these conditions are partially saturated. The T_1 for Pi is 8-10s and for intracellular ATP is between 300-500 ms. Correction factors were estimated for the extracts by collecting spectra from one extract with a 50 s repetition rate (i.e. $> 5 \times T_1$ of metabolites of interest).

Peak areas were estimated using MACFid curve fitting software (Tecmag, Houston, TX, see Appendix 3) or standard Bruker integration software.

6.1.4 Statistics

Cell and MRS studies were repeated 3-4 times. Data are presented as mean $\pm$ one standard error of the mean for the cell results and mean $\pm$ one standard deviation for the MR results. Means are compared using the 2-sided unpaired t-test

6.2 Results

6.2.0 Characterization of Cells Embedded in Alginate

Calcium alginate beads were 1.8 ± 0.2 mm in diameter. Immediately following preparation, cells were evenly dispersed throughout the beads. Two days following encapsulation beads show a rim of growing cells and the central core contains dying cells (Figure 6.3). Note the spheroid shown in Figure 6.3 shrank following fixation. This necrotic fraction accounts for less than 10% of the cells as estimated from the histological sections.

With only four time points it is not possible to accurately model the population growth inside the alginate beads. Cell numbers doubled between the first and second day post encapsulation (see Table 6.1) after an initial cell loss between days zero and one post-plating.

Table 6.1 Relative increase in cell numbers of cells entrapped in calcium alginate beads.

Time in alginate beads (days)	Number of cells per bead ^ξ
0	1.0
1	0.83 ± 0.11
2	1.63 ± 0.24
3	1.87 ± 0.36

^ξ relative to initial population ($\sim 3 \times 10^5$ cells/bead)

Figure 6.3 Section through a calcium alginate bead containing A2780 ovarian cancer cells two days following encapsulation.

By the third day the pH of the medium was becoming acidic and the nutrients had been depleted. Hence the growing conditions were suboptimal

Figure 6.4 shows the surviving fraction of the A2780 and 2780^{CP} cell lines as a function of time post encapsulation. Survival of the cells (relative to the unencapsulated cells and averaged over both the A2780 and 2780^{CP} cell lines) decreased to $76\% \pm 12\%$ following encapsulation and immediate retrieval. After 24 hours the cells had returned to $95\% \pm 9\%$ of their original plating efficiency and $87\% \pm 6\%$ after 48 hours. The survival had decreased to $60\% \pm 7\%$ after 72 hours of encapsulation in beads.

Three repetitions of flow cytometry measurements were made on the A2780 cell line and for comparison a single repetition of the 2780^{CP} cell line was performed. With the exception of the 72 hour point, the cell cycle distribution of the 2780^{CP} cell line is qualitatively similar to the A2780 cell line (see Figure 6.5). In late exponential (prior to encapsulation) the percentage of G₁/G₀ A2780 cells is $85.7 \pm 3.4\%$. This drops to $54.9 \pm 6.5\%$ after 24 hours and rises slightly over the next 48 hours. The percentage of S phase cells increases from $7.6 \pm 1.3\%$ to $28.3 \pm 0.1\%$ during the first 24 hours and decreases back to $20.4 \pm 4.7\%$ as the media conditions become less conducive to growth. These analyses indicate that a subpopulation of cells in alginate are progressing through the cell cycle.

6.2.1 ³¹P MRS of Perfused Intact Cells.

The survival fractions of cells entrapped in alginate and transported on ice to the MR facility were $78\% \pm 9\%$. This loss in survival was comparable to the difference in survival observed when cells were released from alginate immediately following preparation (see Figure 6.4). Thus, transportation on

Figure 6.4 Plating efficiency of two ovarian cancer cell lines (A2780 and its cisplatin resistant mutant 2780^{cp}) as a function of time encapsulated in calcium alginate beads.

Figure 6.5 Cell cycle distribution of two ovarian cancer cell lines (A2780 and the cisplatin resistant mutant 2780^{cp}) as a function of time encapsulated in calcium alginate beads.

ice did not significantly alter cell survival. Spectra of perfused wild type ovarian cancer cells and the cisplatin resistant mutant are shown in Figure 6.6. Concentrations of these metabolites are given in Table 6.2. Relaxation times of ^{31}P metabolites in intact cells are not the same as those in solution, hence the ratios are not corrected for partial saturation effects. The cisplatin resistant mutant exhibits elevated levels of PCr compared to the sensitive parental cell line. The ratios of Pi/ β -ATP were not estimated because the perfusate contained Pi.

Table 6.2 Relative levels of phosphate metabolites in perfused intact ovarian cancer cells.

Cell Line	PCr/ β -ATP	PME/ β -ATP	PDE/ β -ATP
A2780	0.15 ± 0.04	0.23 ± 0.05	0.03 ± 0.01
2780CP	$0.28 \pm 0.03^{\xi}$	0.23 ± 0.03	$0.13 \pm 0.04^{\xi}$

ξ Ratio significantly different from ratio observed for parental cell line.
($P < 0.05$)

6.2.2 ^{31}P MRS of Cell Extracts

In order to check the assignments of metabolites in the ^{31}P MR spectra perfused cells, spectra of PCA extracted ovarian cancer cell lines (wild type, adriamycin resistant mutant and cisplatin resistant mutant) were measured and are shown in Figure 6.7. The adriamycin resistant cell line was included to allow our findings to be compared with previously published reports. The

Figure 6.6 ^{31}P MR spectra of two ovarian cancer cell lines a) A2780 (sensitive parent) and b) 2780cp (cisplatin resistant mutant) encapsulated in calcium alginate beads and maintained in a cell perfusion system

Figure 6.7 ^{31}P MR spectra of perchloric acid extracts of three ovarian cancer cell lines a) A2780 (sensitive parent), b) 2780^{cp} (cisplatin resistant mutant) and c) 2780^{ad} (adriamycin resistant mutant).

concentrations of acid soluble phosphate metabolites (PCr, PME and PDE) are given in Table 6.3. These areas of the resonances from these metabolites are presented relative to β -ATP to facilitate the comparison of levels with those observed for the perfused intact cells (Table 6.2). These concentrations were corrected for partial saturation effects. Both resistant cell lines had significantly higher ratios of PCr/ β -ATP and differences in specific sugar phosphates and phosphomonoesters (see Figure 6.7). The cisplatin sensitive cell line had a significantly increased ratio of PDE/ β -ATP.

Table 6.3 Relative levels of phosphate metabolites from perchloric acid extracted ovarian cancer cells.

Cell Line	PCr/ β -ATP	PME/ β -ATP	PDE/ β -ATP
A2780	0.33 ± 0.08	1.41 ± 0.20	0.17 ± 0.01
2780 ^{CP}	$0.75 \pm 0.10^{\xi}$	1.23 ± 0.31	$0.41 \pm 0.12^{\xi}$
2780 ^{ad}	$0.62 \pm 0.10^{\xi}$	1.41 ± 0.46	0.28 ± 0.07

ξ Ratio significantly different from ratio observed for parental cell line (P<0.05)

6.3 Discussion

Cancerous cells often exhibit uncontrolled growth. A treatment does not necessarily have to kill cells to be successful, it may be sufficient to simply stop cell division. The loss of reproductive ability can be monitored by the clonogenic assay. ^{31}P MRS provides quite different information. With cells

that have received a dose which results in <1% clonogenic survival, a temporary improvement in overall bioenergetic status has been observed (102). MR provides information on the metabolic pathways used and on different and changing control of these pathways. It is this information which has potential for providing some insights into differences in energy requirements of sensitive and resistant cells. However, one important endpoint is whether or not the cells can divide. It is thus important to be able to correlate the MR measurements with the total cell numbers being studied and the clonogenic potential of the population.

With this study we showed that cells incubated in alginate beads were able to proliferate and remain clonogenic for at least 72 hours. This period is comparable to the length of time over which Ng *et al.* (102) observed changes in the ^{31}P MR spectra of RIF-1 mouse fibrosarcoma cells in alginate treated with γ -radiation. Cells in the outer 400 μm rim of the alginate beads were able to proliferate. After a 24 hour lag period the cell numbers approximately doubled. This lag period was also observed for the growth curves of these cells on tissue culture dishes (Chapter 3). In a study with EMT6/Ro cells entrapped in alginate beads (~2.6mm diameter) it was shown that 96% of cells experience oxygen tensions of $>20\mu\text{M}$, i.e the hypoxic fraction was very small. The necrotic region in the centre of the beads is therefore possibly due to an inadequate supply of nutrients or to waste products generated by the cells not being removed. The percentage of cells in the necrotic region of our beads was <10%. Studies with spheroids have shown that with this size necrotic zone there is no impact on the energy metabolites measured using ^{31}P MRS (101). However, following treatment, up to 50-90% of these cells may be dying and the byproducts of cell lysis may accumulate within the beads.

By the third day the pH of the medium was down to 6.7, resulting in reduced cellular proliferation, DNA synthesis and glycolysis (112). Thus, the cell numbers did not increase significantly between days two and three post-encapsulation.

Cells entrapped in alginate were able to retain approximately 90% of their clonogenic potential for 48 hours. This differs from the results of Ng *et al.* (102) who found that the RIF-1 cell line did not remain clonogenic in alginate. This may be accounted for by the high cell densities used for MR studies (5×10^7 cells/cm³). When supplied with adequate nutrients, the A2780 and 2780^{CP} cell lines continue to divide even after they have reached confluency. It is probable that the clonogenic potential of the A2780 cell line is not impaired by the high cell numbers.

The cells can proliferate in alginate. On day zero, most of the cells are in the plateau phase whereas on day two a larger subpopulation are growing exponentially. Thus, the population assayed on day two is not the same population as on day zero and differences in the plating efficiencies are to be expected. MR studies observe the entire, changing population. If the metabolic responses of treated cells and their progeny are to be correlated to the survival of the population, it is imperative that an untreated control of entrapped cells be exposed to the same conditions as those undergoing treatment and that the survival of treated cells be normalized to their untreated counterparts.

The initial drop in plating efficiency observed immediately following entrapment may be explained by the effects of trypsin and the entrapment procedure. The return of the plating efficiency to its original level after 24 hours probably reflects the recovery of cells following these manipulations and the adaptation of cells to the spinner flask.

Cell cycle analyses are in agreement with the observed increase in cell numbers. Cells harvested on the eighth day post plating are in late exponential, early plateau phase. After 24 hours in alginate, circulating in fresh medium, the percentage of S phase cells increased indicating that some cells have started to proliferate. After three days in medium the percentage of S phase cells is beginning to drop as could be predicted from the low medium pH.

Some recommendations can be made for studying treatment protocols using this system. Firstly, this system models a growing tumour, with a necrotic centre and proliferating cells on the surface. If a more homogeneous population is required/desired, cells can easily be embedded in alginate threads, rather than beads, which have a diameter of $<0.5\text{mm}$. To ensure that different cell lines and cells treated with different doses are studied under similar metabolic conditions, it may be necessary to use fresh medium throughout the MR measurement. This would ensure that waste products from treated cells are removed and that cells that are able to proliferate can do so.

These changes were not necessary for the much more limited study undertaken for this thesis. Cells were not treated and were only observed by MRS for 3-4 hours.

Increased ratios of PCr/ β -ATP were observed in both perfused and PCA-extracted adriamycin- and cisplatin-resistant cells compared to those of the parental cell line. PCr is a subsidiary means of energy storage for anaerobic glycolysis. A higher level of PCr could increase the capacity of cells to maintain their ATP pool (73). The cisplatin-resistant cell line is known to have increased DNA repair capacity (64) and reduced drug accumulation (63). The mechanism via which cisplatin is taken up by the cell is still unknown; however, changes in drug uptake and DNA repair may result in different energy requirements of the cell. It is interesting that primary resistance to both

adriamycin and cisplatin, which are very different classes of drug, both result in elevated PCr. This observation could possibly be exploited in the early prediction of resistance. In agreement with the extract results, the PCr/ β -ATP level of the perfused 2780CP cell line was also higher than in the sensitive parental line. However, the ratio was lower in the intact cells compared to the the extracts. Part of this difference is, of course, that the ratios were not corrected for partial saturation effects. However, an additional concern was that the level of PCr declined as a result of transport of cells on ice from the ORCC to the NRC.

Additional differences in control of metabolic pathways have already been observed for adriamycin-resistant cancer cells (72,73). GPC and GPE, breakdown products of phospholipids, are an indicator of phospholipase activity. Our adriamycin-resistant cell line had unchanged levels of total PDEs and PME, although differences in the specific sugar phosphates and phosphomonoesters were observed. These results are consistent with the ^{31}P MR study of adriamycin -sensitive and -resistant human small cell lung carcinoma cell lines which found no differences in levels of PME and PDE between the two cell lines (73). By contrast, PME and PDE levels were decreased in adriamycin resistant MCF-7 breast cancer cells (72). Thus, differences between the levels of these metabolites in adriamycin -sensitive and -resistant cell lines are not consistent from one cell line to another and therefore do not show promise as markers for predicting adriamycin resistance. The cisplatin resistant cell line, consistent with our findings in Chapter 3, exhibited an increased ratio of PDE/ β -ATP. The universality of this finding is also arguable since an increased PDE level was not observed in the ^1H MR results of the 2008/C13* ovarian cancer cell lines pair (Chapter 3)

in the introduction it was discussed that ^{31}P MRS can go beyond identifying therapy resistance. Even prior to treatment, ^{31}P MRS detects increased levels of PCr in the resistant cell lines compared to the sensitive parental cell line. If energy consuming processes are partly responsible for drug resistance it is likely that differences in the energy consumption following treatment will be reflected in the spectra. Thus, the power of this technique is that it can be used to monitor the early response of cells to treatment. Future studies in this direction are discussed in the following chapter.

6.4 Conclusions

Cells entrapped in alginate model a growing tumour. A small necrotic core is surrounded by proliferating cells. These cells can be retrieved during MR studies to measure their clonogenic potential.

The perfusion system was designed to incubate the alginate beads in a controlled environment. The temperature and drug concentration in the medium can be rapidly changed in this system.

The difference between cisplatin -resistant and -sensitive ovarian cancer cells was examined in this system and compared to results obtained from extracts of these cells and an adriamycin resistant cell line. Both cell lines exhibited increased PCr/ β -ATP ratios. The cisplatin resistant cell line had an elevated PDE/ β -ATP ratio.

This system can potentially be used to see whether the early response of cells to a given course of treatment is a better predictor of outcome than the untreated spectrum.

CHAPTER 7   SUMMARY AND FUTURE DIRECTIONS

7.0   Summary and Conclusions

At the beginning of this thesis we presented two aims. The first was to characterize ovarian cancer using ^1H MRS. An end point of this study was to evaluate whether ^1H MRS had potential for screening women for ovarian cancer. Cure rates for Stage I ovarian cancer are high (over 90%) whereas the 5-year survival rates for advanced ovarian cancer have not improved in the past 20 years and remain less than 20% despite advances in chemotherapy. Thus, detecting cancer early would have a marked impact on the mortality rate. Since ovarian cancer is asymptomatic in its early stages, and it is at this point that cancer detection would have the most benefit, screening would have to be performed on asymptomatic women.

Given the scarcity of health care funds, screening should be limited to patients at higher risk. Two risk factors are age (> 50 years) and a family history of the disease (123). Screening these high risk women can be done more cheaply by a combination of abdominal ultrasonography, measurement of the serum levels of CA 125 (an antigenic epitope carried on a high molecular weight glycoprotein) or annual pelvic examination. However, elevated serum levels of CA 125 can result from benign gynecological problems and ultrasonic screening cannot reliably distinguish between benign and malignant ovarian tumours (123). Clinical use of MRS in combination with MRI can provide detailed biochemical information about the tumour in addition to tumour detection.

This leads to the second aim of this thesis which was to determine whether ovarian cancer cells which were resistant to drug treatment could be

distinguished from cells which respond well to treatment on the basis of the biochemical profiles measured using ^1H MRS. The clinical importance of this part of the study is that if patients who have resistant tumours can be identified prior to treatment, these patients may be given alternative or more aggressive treatment regimens.

To evaluate the potential of ^1H MRS in characterizing ovarian cancer, we examined three models of human ovarian cancer. Starting with the simplest model, two human ovarian cancer cell lines from untreated patients were grown to confluency in monolayers and examined using 1D and 2D COSY ^1H MRS. Predominant resonances in the ^1H spectra of these cell lines were triglyceride methyl, methylene, total choline, total creatine, soluble membrane products and amino acids. These resonances were also observed in spectra obtained from *ex vivo* ovarian cancer. Metabolic profiles of ovarian cancer cells derived from different patients exhibited significant variations in spite of controlled growing conditions.

However, even within a tumour, growing conditions can vary, depending on the distance from the nutrient supply. Over 90% of the tumours obtained for this study contained areas of necrosis (although the pathologist attempted not to include necrotic specimens for MR analysis). Parts of the tumours are actively growing whereas others are quiescent or dying. Given the range of these growth states, we returned to the simple cell model to examine the dependence of the ^1H MR spectrum on the growth phase. Exponentially growing cells harvested from monolayer cultures exhibited attenuated lipid signals and elevated phosphomonoesters (which are associated with membrane synthesis) compared to cultures harvested in plateau phase. Exponentially growing cells grown as spheroids in which the cells are packed at tissue-like density have spectra which were not significantly different from those grown

in monolayers, indicating that for this cell line, packing density does not have a pronounced influence on the observed spectrum

The spheroid model was also used to assess the importance of necrotic areas on the measured spectrum. The percentage of necrotic cells correlated well with the amount of mobile lipid observed in the ^1H spectra. Differences in the amount of mobile lipid have been used to distinguish normal ovarian tissue from ovarian cancer and, in this study, were identified as being an important parameter for distinguishing between untreated and recurrent ovarian cancer. This may mean that we are separating classes, in part, on the amount of necrotic tissue present.

In this study we also compared the ovarian cancer cell lines obtained from untreated patients to mutants of these cell lines in which resistance to the chemotherapy drug cisplatin was induced. Differences between the resistant compared to the sensitive cell lines included changes in the amount of mobile lipid, increased levels of glutathione and taurine and decreased levels of glutamate. These cell lines were 7-20 fold resistant and the level of resistance observed clinically is 2-10 fold (28). Although it is not known which mechanisms are primarily responsible for clinical resistance, elevated glutathione levels, increased DNA repair and changes in uptake have been demonstrated in humans (28) and these mechanisms have been identified as contributing to resistance in the cell lines we examined (79). Thus the differences we observed between drug -sensitive and -resistant cell lines will help in interpreting the differences we observed between untreated and recurrent ovarian cancer samples (*ex vivo*).

^1H MR spectra of normal ovarian tissue and ovarian neoplasms obtained during surgery were measured. These specimens contained a heterogeneous population of cells which lead to large variations in the ^1H spectra. Many of

these variations did not contribute any information of diagnostic interest. Using Linear Discriminant Analysis we were able to reduce the number of variables which were important for discriminating between normal ovarian tissue and ovarian cancer to six. The chemical shifts of these spectral attributes were consistent with the chemical shifts of lysine, creatine, choline and taurine, $=CH_2-CH_2-CH=$ and $-OOC-CH_2-CH_2-$. However, given the uncertainty of the chemical shifts following data-compression, these assignments are not definitive. Using these attributes, ovarian cancer could be distinguished from normal ovarian tissue with a sensitivity of 100% and a specificity of 95%. This showed an improvement over the three parameter classification technique reported by Mackinnon *et al.* (31). Interestingly, different samples were misdiagnosed by the two techniques. A more robust diagnosis of ovarian cancer can be made using a combination of classification techniques which can be used to classify samples by consensus (11).

LDA was then applied to the subgroup of treated and untreated serous and endometrioid ovarian cancers. On the basis of ten attributes, untreated ovarian cancer could be distinguished from treated cancer with a sensitivity of 92% and a specificity of 100%. The predominant metabolites consistent with the chemical shifts of these attributes are triglyceride methyl, methylene, $-CH_2-CH_2-CH=$, glutamate, $=CH-CH_2-CH=$, choline, taurine, glutamine and/ or glutathione. Several of these metabolites were identified as distinguishing between drug -resistant and -sensitive ovarian cancer cells in Chapter 3. Interpretations of these findings include a) recurrent tumours contain cells which were either intrinsically resistant or which developed resistance following exposure to chemotherapeutic agents and these cells exhibit changes in their ^1H MR spectra consistent with the differences observed in *in vitro* resistant cells, b) chemotherapy induces changes in ovarian cancers but some

of these changes may not necessarily be indicative of specific resistance mechanisms and c) the differences observed between recurrent and untreated ovarian cancers may simply represent differences between primary and secondary tumours. One would also expect some of the untreated tumours to be intrinsically resistant to chemotherapy. With relapse times of up to 10 years it is difficult to assess this possibility (see future directions). Nonetheless, ^1H MRS shows promise in characterizing the properties of ovarian cancer cells which are resistant to chemotherapy.

Using ^{31}P MR to monitor energy and phospholipid metabolism of viable cells, further differences between sensitive and resistant ovarian cancer cells were observed. The system used to obtain these results can be used to measure the early response of cells to treatment and has the potential to be used to optimize treatments on resistant cell lines (see future directions).

7.1 Future Directions

The resistant human ovarian cancer cell lines examined in this thesis had their resistance induced *in vitro* and the level of resistance was somewhat higher than that observed clinically. The mechanisms of resistance in these cell lines may not fully model what is observed clinically. Taurine has not been implicated as a mechanism of resistance but it does have important protective roles to play in cells. It would therefore be interesting to establish whether the level of resistance conferred on cells is related to the level of taurine and further, whether taurine assists in membrane stabilization and drug detoxification in resistant cells. In this study, the ovarian tumours exposed to cisplatin *in vivo* do not exhibit elevated levels of glutathione or taurine. Reasons for this include a) the clinical level of resistance is lower than in the

in vitro induced cell lines and therefore the metabolic changes may not be observable by MRS, b) the mechanisms of resistance are different (either because these tumours were intrinsically resistant or because resistance was induced *in vitro*).

To elucidate this further, cells could be exposed to cisplatin with the same exposure schedule as done clinically. Cells could be examined at intermediate levels of resistance and changes in MR parameters could be correlated to cell survival studies. This study could establish whether low levels of resistance can be detected by MRS. It would also be of interest to determine whether, in an *in vitro* system, changes in the MR characteristics precede, parallel or follow the onset of resistance. To evaluate whether different mechanisms are manifest in cells with *in vivo* induced resistance, cell lines could be established both from patients who are intrinsically resistant (i.e. their tumours grow during treatment) and from patients whose tumours initially respond to treatment but fail to respond to subsequent treatment. These cell lines would lead to some understanding of the mechanisms of "intrinsic" and "acquired" resistance. To this end we have established a cell line from a patient undergoing combination drug therapy including cisplatin. The MR spectra of a biopsy from this patient and the cell line obtained from this biopsy are shown in Figure 7.1. Comparison of these spectra could provide some information on whether drug-resistance mechanisms of cells grown *in vitro* are the same as those of cells from biopsy samples.

In Ottawa, only about 30 cases of ovarian cancer are diagnosed each year. Of these, many are not suitable for collection. However, to fully exploit multivariate techniques, large numbers of samples are required in each class. MR spectra of ovarian cancers obtained in other studies (31) can be combined with this data base to increase the sample size. With more samples, it may be

Figure 7.1 Comparison of the ^1H MRS spectra from a biopsy obtained from an ovarian cancer patient during treatment and cells from the cell line developed from the same biopsy sample. These spectra were measured at 37°C on a Bruker AMX600. The volume of the biopsy sample was larger than the volume of cells resulting in a higher SNR for the biopsy sample.

possible to classify the different histological types and grades of ovarian cancer. Although this may not be of great clinical significance, it will further define the boundaries, in multivariate space, between normal ovarian tissue and the different types of ovarian cancer.

Of more clinical relevance is establishing whether MR can detect chemotherapy-induced changes. A limited number of recurrent ovarian cancer samples (~3 samples per year) was obtained because second-look laparotomies are not routinely performed and surgery on recurrent tumours is only done for debulking or reversal of bowel obstruction. However, ascites fluid is routinely removed from patients who have suffered a recurrence. This fluid can be obtained both from primary and recurrent tumours and may offer valuable diagnostic information.

A second complication arises because ideally the control for the recurrent tumour (or ascites fluid) should be the primary tumour (or ascites fluid) from the same patient. Recurrences can occur many years after the initial diagnosis and thus a long term study would be required. It will also be necessary to correlate spectra obtained from patients prior to chemotherapy to the long term outcome of these patients. This will establish whether the subset of patients whose disease recurs can be distinguished from those whose therapy was successful. A retroactive study of this type would help elucidate whether there are any MR observable differences between "induced" and "acquired" resistance. At this stage another multivariate technique, cluster analysis, could be employed to determine whether there is any natural clustering of the samples in multivariate space.

The classification between untreated and recurrent tumours may arise because recurrent tumours may have grown from metastases that were not removed during the original surgery. An important control will be to determine

whether there are any differences in the MR characteristics between the metastases and the primary ovarian tumours prior to treatment, and between those of untreated metastases and recurrent tumours.

The perfusion system can be used to assess the feasibility of using MR as an indicator of the efficacy of treatment. Cells can be embedded in alginate or grown as multicellular spheroids and the response to combinations of treatments (radiation, drugs and possibly hyperthermia) can be monitored for up to 72 hours using ^{31}P MRS. Measurements of phosphorus-containing metabolites can be correlated to changes in clonogenic survival fraction, cell growth, cell size and cell cycle distribution. Differences in the early response of sensitive and resistant cells to treatment (be it by drugs, radiation or combination therapy) may provide further insight into cellular mechanisms of resistance.

Finally, the suppression of motional artifacts in MR spectra obtained from the pelvis, and in particular the ovary, is actively and successfully being pursued (L. Friesen, personal communication). Many of the studies suggested on biopsy samples can therefore be performed *in vivo*. With this final step, the MR characterization of ovarian cancer will have proceeded from "bench to bedside".

APPENDIX 1 Window Functions for COSY Spectra of Ovarian Cancer Cells

Metabolites in biological samples exhibit a wide range of T_2 relaxation times (124,125). As discussed by Delikatny *et al.* (74), the signal envelope for the cross peaks between two coupled spins is

$$S(t_1, t_2) = \exp(-t_1 / T_2^e) \sin(\pi t_1) \exp(-t_2 / T_2^d) \sin(\pi t_2) \quad \text{a1-1}$$

where T_2^d and T_2^e are the true T_2 times in the detection (d) and evolution(e) periods.

Time domain data measured for 2D COSY acquisitions can be multiplied by window functions either to enhance resolution or to optimize sensitivity. To emphasize shorter T_2 components ($T_2 < 300\text{ms}$), for instance lipids, a window function must be chosen which selects signal from the initial part of the signal envelope. Conversely, for long T_2 components, a window function that covers the entire domain should be used. As suggested by Delikatny *et al.*, the optimal set of window functions may vary from one biological sample to another, and depend on the metabolite of interest. Two window functions were evaluated on the COSY data from one of the cell lines (2008). The acquisition parameters for these cell lines were as follows: spectral width in the frequency domain F2 ($\equiv \omega_2$ domain in Figure 2.8) 2824 Hz, 2K t_2 time domain points, (with a dwell time of 177 μs for a total acquisition time in $t_2 = 362\text{ ms}$), 160 t_1 time domain points, (dwell time = 354 μs). For processing, the t_1 domain was zero filled to 1K. One of the following window functions was then applied and the data were Fourier transformed in both dimensions. The resulting spectra were symmetrized and are presented in magnitude mode.

Details on the acquisition and processing of COSY data can be found in references (48,74).

The first window function was the sinebell function

$$\text{SB} = \begin{cases} \sin\left(\frac{\pi t}{T}\right) & t < T \\ 0 & t > T \end{cases} \quad \text{a1-2}$$

where T was varied from 91 ms to 362 ms, i.e. for a 2K file, the time domain size (TD2 in Bruker terminology) was varied from 512W to 2K

The second window function was the Lorentzian-Gaussian transformation

$$\text{LG} = \exp[-\pi \cdot \text{LB}(t - \frac{t'}{2})] \exp[\pi \cdot \text{LB} \cdot \frac{t'^2}{2t'}] \quad \text{a1-3}$$

where LB is the line narrowing in negative hertz and t' is the time at which the height of the Lorentzian function is maximized (in Bruker terminology $t' = \text{GB} \times \text{AQ}$). LB was varied from -30 Hz to -40 Hz. AQ was constant (= 362 ms) and t' was varied via the GB command from GB = 0.12 to 0.24. These functions are shown for reference in Figure A1-1.

Examples of four window functions are shown in Figure A1-2. The resolution of the metabolites was greatest in figures A1-2a, A1-2b using sinebell functions over the entire time domains [TD2 = 2K, TD1 = 160] or the Lorentz - Gauss function [LB -30, GB .24]. However, fewer metabolites were visible. The Lorentz - Gauss function [LB -40, GB .12] offered no benefit over the sinebell function [TD2 = 512W, TD1 = 160]. The parameters chosen were a sinebell over 160W in the t_1 domain and a shortened sinebell (512W) in the t_2 domain.

Figure A1-1 Time domain window functions

a)

b)

3 of / de 3

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISION^{SM} RESOLUTION TARGETS

Figure A1-2 Contour plots of COSY spectra from the ovarian cancer cell line 2008 processed using different window functions a) sinebell functions over the time domains TD2 = 2K, TD1 = 160 b) Lorentz - Gauss function [LB -30, GB .24] c) sinebell function TD2 = 512W, TD1 = 160 and d) and Lorentzian -Gaussian function [LB -40, GB .12] (using the conventions described in the text).

APPENDIX 2 Baseline and Phase Correction

Two of the sources of error discussed in the theory section (Chapter 2) are phasing and baseline correction. Variations in preprocessing spectra can lead to large differences in the peak amplitudes measured. Changes in measured peak amplitudes as a function of phase and baseline correction were assessed. In addition, the signal-to-noise ratio of the choline peak was measured (after exponential line broadening)

Firstly, a 1D spectrum of ovarian cancer cells was phased. Initially the spectrum was phased to maximize the amount of positive signal in the real spectrum using software written in-house at the Institute for Biodiagnostics, National Research Council. Using the processing software, MacFID (Tecmag, Houston, Tx.), zeroth order phase corrections were made to this spectrum (-15° to 15°) and the amplitudes of selected peaks (at 0.9, 1.3, 2.0, 3.0, 3.2 and 3.9 ppm) were measured. These amplitudes, expressed as a percentage of the spectral amplitudes of the spectrum phased initially, are presented in Figure A2-1. Phase corrections of more than 10° resulted in extremely distorted line shapes. Misphasing the spectra by up to 10° can result in changes in the peak amplitudes of, in the worst case (the $-\text{CH}_2-$ peak), up to 13%. Other peaks within the region of interest varied by less than 5%.

Baseline correction was performed on the optimally phased spectrum. This was done in two ways. The first was to spline fit to 11 points spread randomly throughout the chemical shift range (11 ppm - 6 ppm, 0 ppm - -3 ppm). The second method was to fit the baseline (in the same chemical shift range) to a 3rd order polynomial. Ten repetitions were made for each method (i.e. spline or polynomial). The standard deviations for selected peaks (as a percentage of the average amplitude) are shown in Table A2-1.

Figure A2-1 Change in the amplitudes of selected peaks (with respect to the amplitude of the automatically phased spectrum) as zeroth order phase corrections were applied to this spectrum (-15° – 15°).

Table A2-1 Change in intensity of selected peaks with baseline correction method

Chemical Shift	Spline Fit Baseline Standard Deviation (%)	Polynomial Fit Baseline Standard Deviation (%)
3.9	8.1	0.8
3.2	6.5	2.3
3	9.7	1.0
2	2.7	1.1
1.3	0.3	0.2
0.9	0.7	1.7

Baseline correction using the a 3rd order polynomial gave more reproducible peak amplitudes. Thus for processing all the 1D ^1H MR cell data, eight chemical shift points were selected and a 3rd order polynomial fit through these points was subtracted from each spectrum to give the baseline-corrected spectra used in Chapters 3-5.

The signal-to-noise ratio (SNR) of a selected peak is given by

$$\text{SNR} = \frac{Y_i(P) - Y_0}{2 * \left[\left(\sum_{i=A1}^{A2} (Y(i) - Y_0)^2 \right) / N \right]^{1/2}} \quad \text{A2-1a}$$

$$Y_0 = \left(\sum_{i=A1}^{A2} Y(i) \right) / (N+1) \quad \text{A2-1b}$$

where $Y(P)$ is the peak amplitude, $A1$ and $A2$ are the chemical shifts between which the noise is calculated, $N = A2 - A1$

The SNR of the choline peak, for example, is 609 ± 77 (where the uncertainty arises from the choice of $A1$ and $A2$ defined above) Thus, the contribution to the uncertainty of the amplitude measurements from noise is small compared to phasing and baseline correction errors

APPENDIX 3 Fitting ^1H and ^{31}P MR Spectra

^1H and ^{31}P MR spectra were fitted to a sum of Lorentzian line shapes using the simulation routine supplied with MacFID (Tecmag, Houston, Tx.). Prior to fitting, the spectra were Fourier transformed, phased (0th and 1st order) and the ^1H spectra were baseline corrected using a 3rd order polynomial. The real spectra were then fitted to Lorentzian line shapes using a non-linear least squares algorithm. The fit parameters are the peak amplitude, half-width at half-maximum and line centre (i.e. the chemical shift). Unfortunately, this routine did not allow *a priori* knowledge to be incorporated into the fit, i.e. if a peak in the chemical shift range of glutamate, for example, were being fitted this software would not automatically take this into account when fitting other resonance peaks of glutamate. This ability is particularly important for the overlapping resonance observed in ^1H spectra. However, even when this knowledge is incorporated (126), errors in the quantification of metabolites in the ^1H chemical shift region between 1.3 and 3 ppm have coefficients of variance over 60%.

As can be seen below in Figure A3-1, the ^1H spectra from drug-sensitive ovarian cancer cells can be fitted to a sum of Lorentzians (although the presence of non-Lorentzian line shapes may account for some of the signal observed in the baseline). However, the same set of Lorentzians could not be used for the resistant pair of this cell line.

By contrast, the well resolved ^{31}P spectra from extracted cells could be fitted to a sum of Lorentzians (see Figure A3-2) and the same set of line shapes could be used for all ^{31}P spectra.

For our studies, it was decided that only the ^{31}P MR spectra should be fitted to a sum of Lorentzians. With the exception of brain MR studies, the

majority of ^1H spectra from cells and tissues is quantified by either integrating resonances or presenting ratios of peak heights (9,10,13, 30, 31) Thus, to facilitate comparison with other studies, we have opted to do the same while acknowledging some of the shortcomings of this approach (78) Finally, using multivariate analyses, regions of the spectra are used as discriminants without measurements of peak heights or areas, obviating the need to fit the spectra by other means.

Figure A3-1 ^1H MR spectrum of drug-sensitive ovarian cancer cell line fitted to a sum of Lorentzian line shapes a) residual, b) Lorentzian line shapes used to fit, c) fitted spectrum and d) original spectrum (n.b. the HOD water peak (4.5-5.5 ppm) was not fitted).

Figure A3-2 ^{31}P MR spectrum of an ovarian cancer cell line fitted to a sum of Lorentzian line shapes a) residual, b) Lorentzian line shapes used for fit, c) fitted spectrum and d) original spectrum

APPENDIX 4 Glutathione Formula and ^1H Spectrum

Glutathione is a tripeptide (γ - Glu - Cys - Gly) found in nearly all cells. The structural formula for glutathione is shown in Figure A4-1. A 1D ^1H MR spectrum of a 5mM solution of glutathione dissolved in PBS/D $_2$ O is shown in Figure A4-2. This was measured at 37° C on a Bruker narrow bore 500 MHz spectrometer

Figure A4-1

Structural formula of glutathione

Figure A4-2 ^1H MR spectrum of 5mM glutathione solution in PBS/D₂O.

APPENDIX 5 Assignment of Cross Peaks in the COSY Spectra of Ovarian Cancer Cells

Prominent cross peaks present in the 2D COSY spectra of cancer cells (8, 31, 75, 116, 127) are given in table A5-1 and Figure A5-1. The coupled protons are indicated in bold face

Table A5-1 Assignment of cross peaks in 2D COSY spectra of cancer cells

Metabolite	Abbrev	Coupling (H)	Chem. Shift F2-F1 (PPM)
<u>Amino Acids and related compounds:</u>			
Alanine	Ala	-CH-CH ₃	1.49-3.79
Aspartate	Asp	-CH-CH ₂ -COO ⁻	4.03-2.89
Glutamate/ Glutamine	Glu/ Gln	CH-CH ₂ -CH ₂ -COOH CH ₂ -CH(N ⁺ H ₃)-COOH	2.21-2.62 2.21-3.81
Histidine	His	CH-CH ₂ -ring	3.22-3.95
Isoleucine	Ile	CH ₂ -CH ₃	0.97-2.12
Leucine	Leu	CH-(CH ₃) ₂	0.97-1.78
Lysine	Lys	CH ₂ -CH ₂	1.72-3.05
Proline	Pro	-αCH-βCH ₂	2.38-4.14
Proline	Pro	-γCH ₂ -βCH ₂	2.11-2.38
Proline	Pro	-γCH ₂ -δCH ₂ -	2.11-3.38
Taurine	Tau	H ₃ N ⁺ -CH ₂ -CH ₂ -OSO ₃ =	3.28-3.44
Threonine	Thr	CH-HOCH-CH ₃	1.33-4.27
Valine	Val	CH-(CH ₃) ₂	1.03-2.34
<u>Membrane Related Compounds:</u>			
Triglyceride	A	CH ₂ -CH ₂ -CH ₃	0.90-1.30
	B	CH ₂ -CH ₂ -CH ₂ -CH ₃	1.33-2.08
	C	CH ₂ -CH ₂ -CH=CH	2.02-5.38
	D	CH=CH-CH ₂ -CH=CH	2.84-5.38
	E	OC-CH ₂ -CH ₂ -CH ₂ -CH ₂	1.33-1.62
	F	OC-CH ₂ -CH ₂ -CH ₂	1.60-2.30

	G	RO-CH ₂ -CH(OR)-CH ₂ -OR	4 12-5 26
	G'	O-CHH'	4 09-4 29
Cholesterol	Z	CH ₃ -C-ring α	0 85-1 52
Phosphoethanolamine	PEth	H ₃ N ⁺ -CH ₂ -CH ₂ -OPO ₃ =	3 20-4 20
Choline	Chol	(CH ₃) ₃ N ⁺ -CH ₂ -CH ₂ -OH	3 50-4 07
Phosphocholine	PChol	(CH ₃) ₃ N ⁺ -CH ₂ -CH ₂ -OPO ₃ =	3 61-4 25
Glycero PC	GPC	(CH ₃) ₃ N ⁺ -CH ₂ -CH ₂ -OPO-G	3 69-4 38
Fucose	Fuc I	-CH-CH ₃	4 27-1 33
	Fuc II	-CH-CH ₃	4 28-1 25
	Fuc IIb	-CH-CH ₃	4 2-1 19
	Fuc III	-CH-CH ₃	4 3-1 41
<i>myo</i> -inositol	Ins	HOCH-HCOH(ring)	3 28-3 64
Other Compounds			
HEPES			3 20-3 88
Polyamines		CH ₂ -CH ₂	1 78-3 06
Lactate Anion	Lac	CH(OH)-CH ₃	1 30-4 12

Figure A5-1 Assignment of triglyceride lipid acyl chains and glycerol backbone connections (A-G) used in Tables 3.4, 3.5 and A5-1

APPENDIX 6 Output Table From the Linear Discriminant Analysis Program Written In-House

The following is the output table from the linear discriminant analysis routine. The value under "matches" gives the fraction of the data set which is correctly classified. The attribute number is related (linearly) to the chemical shift. Starting with $n = 1$, and increasing to $n = 12$, the best combinations of n attributes are found. In this example, the two classes could be separated with high accuracy using ten attributes. Additional attributes did not improve the classification

Region finding output (DSCR)

Best 1 attribute:

Number	Matches	Attributes
1	0.1753	47

Best 2 attributes:

Number	Matches	Attributes
1	0.4047	29 6
2	0.4049	29 35

Best 3 attributes:

Number	Matches	Attributes
1	0.4854	28 29 36
2	0.5039	29 36 42
3	0.5880	29 35 62

Best 4 attributes:

Number	Matches	Attributes
1	0.6466	2 3 47 104
2	0.5658	2 3 47 91
3	0.6963	29 36 42 47
4	0.5875	2 3 48 105
5	0.6095	29 35 67 68

Best 5 attributes:

Number	Matches	Attributes
1	0.7271	2 3 23 47 91
2	0.7271	2 3 47 80 103
3	0.6713	2 3 35 47 90

Best 6 attributes:

Number	Matches	Attributes
1	0.7828	29 35 44 46 73 85

Best 7 attributes:

Number	Matches	Attributes
1	0.8109	29 35 44 45 46 73 85

Best 8 attributes:

Number	Matches	Attributes
1	0.8109	29 35 44 45 46 64 73 85

Best 9 attributes:

Number	Matches	Attributes
1	0.8110	29 35 44 45 46 57 64 73 85
2	0.7825	9 23 28 47 48 49 62 75 86

Best 10 attributes:

Number	Matches	Attributes
1	0.9004	9 23 28 47 48 49 62 75 86 105

Best 11 attributes:

Number	Matches	Attributes
1	0.9004	9 21 23 28 47 48 49 62 75 86 105

Best 12 attributes:

Number	Matches	Attributes
1	0.8695	9 14 21 23 28 47 48 49 62 75 86 105

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