Understanding the Molecular Mechanism of Collateral Sensitivity of MDR Tumour Cells Expressing ABC Proteins

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Thesis Abstract

Multidrug resistance (MDR) in cancer is a phenomenon that impairs efficient treatment that has now become a major hinderance to chemotherapy. ABC transporters such as the P-glycoprotein (P-gp or ABCB1) and the multidrug resistance-associated protein 1 (MRP1 or ABCC1) are known to actively efflux chemotherapeutic agents out of cells thereby inducing MDR. A promising approach in order to specifically target MDR cells is to take advantage of their increased sensitivity (collateral sensitivity) to certain compounds.

The second chapter of this thesis aims to elucidate the mechanism of collateral sensitivity in MRP1 cells. Overall, the results show a hypersensitivity of these cells to glutathione (GSH) modulating agents. Such hypersensitivity in cells is caused by the presence of MRP1, which extrudes GSH out of the cell. Collateral sensitivity can be achieved by stimulating the extrusion with verapamil (Vrp), Apigenin (Api) or by inhibiting the GSH synthesis with butathione sulfoximine (BSO). Finally, GSH depletion leads to reactive oxygen species (ROS) accumulation, which likely triggers apoptosis.

The third and fourth chapters aim toresolve the collateral sensitivity mechanism induced in P-gp expressing cells. The study of Vrp, progesterone (Pro) and deoxycorticosterone (DOC) induced collateral sensitivity lead to the conclusion that two requirements ar necessary for validating this phenomenon. The first requirement, common to the three compounds, is the presence of and active P-gp. This P-gp sees its ATPase activity stimulated and the electron transport chain higher demand generated leads to the formation of higher levels of ROS. It seems that these ROS alone are not sufficient and they need to potentiate something else. In the case of Vrp it seems that increase Ca^{2+} level would be the second requirement. For Pro and DOC, the presence of cholesterol at the membrane levels seems to be enough. This allows the detection of

collateral sensitivity by both Pro and DOC in P-gp expressing human cell line, a thing that is not found with Vrp.

In conclusion, the elucidation of collateral sensitivity mechanisms could potentially be usefull to direct the development of specific anticancer drugs that would target directly MDR cells.

Résumé Général

La résistance multiple à la chimiothérapie (MDR) dans le cancer est un phénomène qui nuit à l'efficacité du traitement et est devenue un important problème. Les transporteurs ABC comme la P-glycoprotéine (P-gp ou ABCB1) et la protéine associée à la résistance multiple 1 (MRP1 ou ABCC1) sont reconnue pour pomper de façon active les médicaments hors des cellules et de ce fait induisent la MDR. Une approche prometteuse visant à cibler spécifiquement ces cellules MDR est de prendre avantage de leur sensibilité accrue (sensibilité collatérale) face à certains composés.

Le second chapitre de cette thèse visait à élucider le mécanisme de sensibilité collatérale chez les cellules exprimant MRP1. Ensemble les résultats montre une sensibilité accrue de ces cellules face aux agents modulant la GSH. Cette hypersensibilité est causée par la présence de MRP1 qui pompe la GSH hors de la cellule. La sensibilité collatérale a pue être obtenue en stimulant le pompage en utilisant la verapamil (Vrp) ou l'Apigenin (Api), ou en inhibant la synthèse de la GSH avec la buthionine sulfoximine (BSO). Finalement, la diminution de la GSH résulte en une accumulation d'espèce réactive d'oxygène (ROS) déclanchant l'apoptose.

Les chapitres trois et quatre étaient dirigés vers la résolution du mécanisme de sensibilité collatérale induite chez les cellules exprimant la P-gp. L'étude de la sensibilité collatérale induite par la Vrp, la progestérone (Pro) et la désoxycorticostérone (DOC) a mené conclusion que deux pré-requis sont nécessaire pour l'obtention du phénomène. Le premier, commun pour les trois composés, est la présence d'une P-gp active. Cette P-gp voit sont activité ATPase être stimulé et l'élévation de la demande de la chaîne de transport d'électrons entraîne la formation de niveau élevés de ROS. Il semble que ces ROS seuls ne sont pas suffisant et ils on besoins de stimulé quelque chose d'autre. Dans le cas de la Vrp il semble qu'une augmentation des niveaux de Ca^{2+} agisse en guise de second pré-requis. Pour la Pro et la DOC, la présence de

cholestérol à la membrane semble être assez. Cela a permis l'observation de sensibilité collatérale autant pour la Pro que la DOC chez une lignée de cellules humaines, chose que l'on ne trouve pas avec la Vrp.

En conclusion, l'élucidation des mécanismes de sensibilité collatérale pourrait être potentiellement utile pour diriger le développement d'agents anticancéreux ciblant spécifiquement les cellules MDR.

Table of Contents

TITLE PAGE I
THESIS ABSTRACT II
RÉSUMÉ GÉNÉRAL IV
TABLE OF CONTENT VI
ACKNOWLEDGEMENTS IX
CONTRIBUTION OF AUTHORS XI
STATEMENT OF ORIGINALITY XII
STATEMENT FROM THESIS OFFICE XIV
LIST OF FIGURE XV
LIST OF TABLE XVII

COMMON ABBREVIATIONS 1
GENERAL INTRODUCTION 2
References 5

CHAPTER 1: LITERATURE REVIEW 6
Cancer 7
History 7
Carcinogenesis 7
Cancer Treament 10
Chemotherapy 11
Drug Resistance 12
ABC Transporters 15
ABC transporters generality 15
P-Glycoprotein 18
P-gp ATPase 21
P-gp in clinical studies 22
P-gp collateral sensitivity 23
Multidrug resistant-associated protein1 25
Clinical significance 27
Collateral sensitivity 28
Modulation of GSH levels in MRP1 expressing cells 28
$_{L}$-S,R-buthionine sulfoximine 28
Apigenin 29
Verapamil 30
Reactive Oxygen Species and Cell Death 32
Calcium and ROS in cell death 32
Glutathione 33
Protective role of GSH 33

Glutathione metabolism 33
GSH transport 34
Role of GSH in cell death 36
Cell death 36
Apoptosis 37
References 39

CHAPTER 2: Modulation of GSH levels in ABCC1 expressing tumor cells triggers apoptosis through oxidative stress 49

Abstact 50
Introduction 51
Materials and Methods 53
Results 58
Discussion 69
Aknowledgements 74
References 75

CONNECTING STATEMENT 1 82

CHAPTER 3: P-glycoprotein (ABCB1) Modulates Verapamil Collateral Sensitivity of Multidrug resistant tumor cells 83

Abstact 84
Introduction 85
Materials and Methods 88
Results 91
Discussion 103
Aknowledgements 107
References 108

CONNECTING STATEMENT 2 111

CHAPTER 4: Collateral sensitivity to steroids directly modulated by hamster and human P-glycoprotein (ABCB1), differences and similarities with verapamil 112

Abstact 113

Introduction 114
Materials and Methods 116
Results 119
Discussion 131
Aknowledgements 135
References 136

GENERAL CONCLUSION 138
References 143
APPENDIX 144

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Contributions of Authors

The majority of the experiments were designed and performed by the author under the supervision of Dr. Elias Georges. The contribution of the co-authors was very important for the completion of these studies. In the first manuscript (chapter 2), Joel Karwatsky (Dr. Elias Georges' previous student) helped in the completion of experiments in figure 1 and 2 as well as for the ELISA in figure 5. Maximilian C. Lincoln (Dr. Elias Georges' previous student) did the Bcl2 transfection and Mara L Leimanis (Dr. Elias Georges' previous student) did the radioactive iodination necessary for figure 6. In the second and third manuscript (chapter 3 and 4) for some experimental replicate, plasma membrane preparation from Jing Lian (Dr. Elias Georges' previous research assistant) figures 5 and 3 respectively). Raghuram Ambadipudi (Dr. Elias Georges' current student) performed the experiment necessary for figure 4 and assisted in the completion of figures 5 to 8.

Statement of Originality

The following components of each manuscript in this thesis contributed original material to the advancement of knowledge in the domain of biochemical cancer research.

Manuscript 1 (chapter 2). Laberge RM, Karwatsky J, Lincoln MC, Leimanis ML, Georges E. 2007. Modulation of GSH levels in ABCC1 expressing tumor cells triggers apoptosis through oxidative stress. *Biochem Pharmacol.* 73(11):1727-37.

The aim of this manuscript was the elucidation of the mechanism of collateral sensitivity of MRP1expressing cells. This study shows this sensitivity of MRP1 cells to GSH modulating agents. It links the GSH extrusion induced by MRP1 and the accumulation of toxic reactive oxygen species leading to apoptosis.

Manuscript 2 (chapter 3). Rémi-Martin Laberge and Elias Georges. P-glycoprotein (ABCB1) Modulates Verapamil Collateral Sensitivity of Multidrug-resistant Tumor Cells. (manuscript in preparation)

In this manuscript the goal was to clarify the role of P-gp in collateral sensitivity to verapamil. It was found that P-gp was essential to the mechanism of collateral sensitivity. In addition the electron transport chain contribution of reactive oxygen species was associated with the toxicity observed. Finally, the limitation of collateral sensitivity phenomenon to certain cells was suggested to be attributed to and increase intracellular levels induced by sorcin expression.

Manuscript 3 (chapter 4). Rémi-Martin Laberge, Raghuram Ambadipudi and Elias Georges. Collateral sensitivity to steroids directly modulated by hamster

and human P-glycoprotein (ABCB1), differences and similarities with verapamil. (manuscript in preparation)

To complement the collateral sensitivity mechanism of P-gp expressing cells, the aim of the third study was to determine the mechanism of collateral sensitivity to steroid hormones. The importance of P-gp in the mechanism was confirmed along with the P-gp ATPase stimulation induced by progesterone and deoxycorticosterone. It was showed that verapamil inhibits this collateral sensitivity and that electron transport chain induction of ROS is also important. The necessity of cholesterol was demonstrate as well, and for the first time, the ability of these compounds (progesterone and deoxycorticosterone) to induce collateral sensitivity in a human P-gp expressing cell line (Mcf7/Adr).

Statement from Thesis Office

As stated in the “Guideline for Thesis Preparation” a thesis may be prepared to include a collection of manuscripts whereby”all components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.”

In addition the thesis must include: “1-a table of contents; 2-a brief abstract in both Englih and French; 3-an introduction which clearly states the rational and objectives of the research; 4-a comprehensive review of the literature (in addition to that covered in the introduction to each paper); 5-a final conclusion and summary; 6-a thorough bibliography; 7-Appendix containing an ethics certificate in the case of research involving human or animal subjects, micro organisms, living cells, other biohazards and/or radioactive material.” Any “additional material must be provided (e.g., in appendices) in sufficient detail to alo a clear and precise judgment to made of the importance and originality of the research reported in the thesis.”

In the event there are co-authored paper “an explicit statement in the thesis” is required “as to who contributed to such work and to what extend.” This is stated in a separate section titled “Contributions of Authors”, and this should appear “as a preface to the thesis.” “The supervisor must attest to the accuracy of this statement at the doctoral oral defence.” This creates difficulty to any examiners and therefore “it is in the candidate’s interest to specify the responsibilities of all the authors of the co-authored papers.”

List of Figures

CHAPTER 1

Figure1. Cigarette consumption and lung cancer **p 9**

Figure 2. Cancer stem cells and drug resistance **p13**

Figure 3. ABC transporters with known drug substrates **p17**

Figure 4. ATP-switch mechanism of ABC transporter **p18**

Figure 5. A hypothetical 2-D model of human P-glycoprotein **p19**

Figure 6. Proposed mechanism of verapamil collateral sensitivity in P-gp1 expressing cells **p26**

Figure 7. Topology of MRP1 **p27**

Figure 8. Redox cycle **p35**

CHAPTER 2

Figure 1. Effects of BSO, VRP or API on the survival of tumor cells with or without ABCC1 expression **p59**

Figure 2. ABCC1 protein expression in tumor cell lines **p62**

Figure 3. Estimates of relative levels of apoptosis, ROS and GSH in H69AR or HeLaABCC1 cells without or with drug treatment **p63**

Figure 4. Reversal of BSO, VRP or API effects on H69AR and HeLaABCC1 cells with GSH or NAC supplemented media **p65**

Figure 5. Relative inhibition of BSO-, VRP- and API-induced apoptosis in H69AR and H69ARBcl2 cells **p66**

Figure 6. Photoaffinity labeling of ABCC1 with IAAGSH **p67**

CHAPTER 3

Figure 1. Verapamil collateral sensitivity in $CH^{R}C5$ clones **p92**

Figure 2. Down-regulation of P-gp1 expression with siRNA **p93**

Figure 3. Effect of Paclitaxel, Doxorubicin or Cis-platinum on the survival of tumor cells with or without P-gp1 expression **p94**
Figure 4. Effect of verapamil on the survival of tumor cells with or without P-gp1 expression **p96**
Figure 5. Total ATPase activity and effects of verapamil on P-gp1 **p98**
Figure 6. Effect of rotenone and Antimycin A on AuxB1 and CH^RC5 cell survival **p99**
Figure 7. Effect of H_2O_2 on CH^RC5 and synergy with verapamil **p101**

CHAPTER 4

Figure 1. Effect of progesterone and deoxycorticosterone on the survival of tumor cells with or without P-gp1 expression **p119**
Figure 2. Effect of PSC833 on drug sensitivity to AuxB1 and CHRC5 **p121**
Figure 3. Effects of progesterone and deoxycorticosterone on P-gp1 ATPase activity **p122**
Figure 4 Effect of verapamil on steroid induced collateral sensitivity **p123**
Figure 5. Effect of rotenone and antimycin A on collateral sensitivity to progesterone and deoxycorticosterone **p125**
Figure 6. Effect of cyclodextrin on CHO cell survival **p126**
Figure 7. Down-regulation of P-gp1 expression with siRNA in human breast carcinomas **p127**
Figure 8. Effect of Doxorubicin, Cis-platinum, Progesterone or Deoxycorticosterone on the survival of tumor cells with or without P-gp1 expression **p129**

List of Tables

CHAPTER 2

Table 1. Effects of GSH modulating drugs on tumor cells growth **p61**

Table 2. Relative effects of GSH modulating drugs, a time-course, on GSH cellular levels, reactive oxygen species and apoptosis in tumor cells expressing ABCC1, relative to control **p64**

List of Common Abbreviations

ABC	ATP binding cassette
Api	Apigenin
BCRP (ABCG2)	Breast cancer resistance protein
BSO	$_{DL}$-buthionine-(SR)-sulfoximine
CFTR	Cystic fibrosis transmembrane conductor regulator
CSC	Cancer stem cell
DOC	Deoxycorticosterone
ETC	Electron transport chain
γ-GCS	γ-glutamylcysteine synthetase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
LTC_4	Leukotriene C_4
MDR	Multidrug resistance
MRP1 (ABCC1)	Multidrug resistance associated protein 1
MSD	Membrane spanning domain
NBD	Nucleotide binding domain
NSCLC	Non-small cell lung cancer
OxPhos	Oxidative Phosphorylation
P-gp1 (ABCB1)	P-glycoprotein 1
Pro	Progesterone
ROS	Reactive oxygen species
SCLC	Small cell lung carcinoma
TM	Transmembrane
Vrp	Verapamil

INTRODUCTION

Cancer is the most deadly disease in developed countries and with the aging of these populations, the number of cases is constantly increasing. Since the introduction of chemotherapy as a treatment for cancer more than 40 years ago, major improvements have been made. While the life expectancies of patients have increased for some cancers, a curative treatment for all cancers remains elusive. The major problem with chemotherapy is drug resistance. Drug resistance is characterized as either intrinsic or acquired. In acquired resistance, tumours that relapse become resistant to treatment. Alternatively, intrinsic resistance occurs in tissues that have an innate tolerance to anti-cancer drugs before initial treatment with chemotherapy. The over-expression of ATP-binding cassette (ABC) transporters has been linked to resistance in many tumours and may correlate with acquired drug resistance as shown by the first selection of multidrug resistant (MDR) CHO cells with colchicine by Juliano et al. (1). These transporters function through the use of ATP hydrolysis to reduce the accumulation of cytotoxic compounds in the cells. Mainly, due to the difficulties in structural analysis of these membrane proteins, the complete mechanism of action is still not understood although hypothetical models have been suggested. Due to their possible implication in clinical drug resistance many efforts were undertaken to develop inhibitors, although the majority of those failed to improve survival rates. This stimulates the requirement for other alternative approaches to undertake this problem. Surprisingly, some multi drug resistant cells show hypersensitivity or collateral sensitivity to other compounds (2). By understand the mechanism of collateral sensitivity, we could take advantage of this phenomenon to specifically target MDR cells.

The multi-drug resistance-associated protein 1 (MRP1 or ABCC1) is an ABC transporter which has been implicated in drug resistance in lung tumours. Interestingly, ABCC1 transports the tri-peptide glutathione (GSH) out of cells.

GSH, a normal cell metabolite, is a major component of mammalian detoxification of reactive oxygen species (ROS). Consequently, as a significant modulator of the cellular redox system, it has been implicated in resistance to oxidative stress induced apoptosis.

Previous studies have shown that a small cell lung carcinoma (SCLC) cell that overexpresses ABCC1 displays a collateral sensitivity to an inhibitor of GSH synthesis (3,4). The drug $_{DL}$-buthionine-(SR)-sulfoximine (BSO) was used to induce this toxicity in ABCC1 expressing cells. Several molecules alter GSH levels in ABCC1 expressing cells; BSO is known to reduce GSH levels in cells, while verapamil (Vrp) is known to promote extrusion of GSH by ABCC1. Apigenin (Api) also causes ABCC1 mediated GSH export. The objectives of the first study (chapter 2) depicted in this thesis were to show that collateral sensitivity in MRP1 expressing cells is induced by the action of GSH extrusion by MRP1 and that this characteristic can be taken advantage of in order to kill these cells using GSH modulating agents.

P-glycoprotein1 (P-gp1 or ABCB1) protein was the first human ABC transporter identified. In addition to the MDR phenotype, certain cells overexpressing this protein show collateral sensitivity to a variety of compounds. Among these compounds, the calcium channel blocker Vrp has been extensively studied, but a full comprehension of the mechanism has yet to be achieved. Although, some evidences sugests that the calcium channel blocker activity is not involved (5,6). Also, the basal P-gp1 ATPase activity stimulation induced by Vrp correlates perfectly with the hypersensitivity (7). Since the phenomenon is not observed in all P-gp1 expressing cells, the direct role of P-gp1 is yet to be confirmed. Furthermore, the possibility of an additional specific feature of these cells leading to hypersensitivity to Vrp remains to be solved. The objective of the second study (chapter 3) is to tackle these two last points.

Some steroid hormones such as Progesterone (Pro) are also known to induce collateral sensitivity in some P-gp1 expressing cells (2). Like Vrp, they stimulate the basal P-gp1 ATPase activity to various levels (8,9). This

hypersensitivity has been less extensively studied and as of now the phenomenon [at least for progesterone (Pro) and deoxycorticosterone (DOC)] was suggested to be similar to Vrp (7). As suggested earlier, it is expected that P-gp1 is not the sole player in collateral sensitivity. It is possible that steroid hormones and Vrp share similarities (e.g. ATPase stimulation) but they may as well have different effect on the cells. Also, it is possible that collateral sensitivity to steroid hormones can be generalized to other cell lines. The objective of the third study (chapter 4) is to address these last two points.

Briefly, the first chapter of this thesis is a summary of the literature focusing on (1) the biology of cancer and its treatment, (2) the implications of ABC transporters focusing P-gp1 and MRP1, and (3) the ROS modulation and implication in cell death. The general objective of this thesis is to characterize the phenomenon of collateral sensitivity in MDR expressing cells, with the hopes that this may provide greater understanding to the translational research of cancer chemotherapy, and ultimately improve treatment outcome for patients. Specifically, the second chapter and first study describes the mechanism of collateral sensitivity of MRP1 expressing cells in the presence of GSH modulating agents. The third and fourth chapters, which encompass studies number two and three, describe insight into collateral sensitivity in P-gp1 expressing cells to Vrp and steroid hormones, respectively.

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Chapter 1

Literature Review

Cancer

History

Cancer was first described by the Greek Hippocrates during antiquity. He called the epithelial tumours he was observing carcinos (crab in English or cancer in Latin) because of their unique shape. Cancer is a disease of cells that affects a wide variety of multicellular organisms. It may affect people of all ages, however the risk for more common cancers tends to increase with age. According to the American Cancer Society, 7.6 million people died from cancer globally in 2007. In 2005, a total of 559,312 cancer deaths were recorded in the United States, which represent about 23% of all deaths in this country (10). The same report estimated a lifetime probability of being diagnosed with an invasive cancer at 45% for men and 38% for women. The nature of cancer suggests that it is a disease of chaos, a breakdown of existing biological order within the body. More specifically, the disorder seen in cancer appears to occur directly from malfunctioning of the controls that are normally responsible for determining when and where cells (throughout the body) will multiply. When the growth limitation controls are deregulated, the cells divide in an anarchic manner leading to a mass called a tumour. Tumours can be either benign (localized, non-invasive) or malignant (invasive, metastatic). The metastases spawned by malignant tumours are responsible for almost all deaths from cancer.

Carcinogenesis

Early epidemiologic studies have shown that the environment is the dominant determinant of the country-by-country variations in cancer incidence. Laboratory research has supported the epidemiologic studies by directly implicating chemical and physical agents (tobacco, coal dust, X-rays) as causes of cancer; tobacco as an example is depicted in figure 1. However, the

possibility of cancer being an infectious disease arose when viruses were found to cause leukemias and sarcomas in chickens. Alternatively, a mechanism that supported carcinogenesis caused by physical and chemical agents surfaced in 1927 when mutations were induced in fruit flies by exposing them to X-rays. By 1950, a series of chemicals were also found to be mutagenic for fruit flies and carcinogenic in laboratory animals. This led to the speculation that cancer was a disease of mutant genes and that carcinogenic agents induce cancer through their ability to mutate genes. In 1975 the Ames test provided support for this idea by showing that many carcinogens can act as mutagens (11).

In organisms, depending on their location and function, cells have different genes either turned on or turned off and are therefore called "differentiated". When one of these cells needs to be replaced, an undifferentiated cell (stem cell) will divide in two. The first will differentiate to replace the missing one while the second cell will remain undifferentiated to maintain a stem cell. This process, called "self-renew", is a hallmark of stem cells. To avoid anarchy, cells must strictly obey cell signals that inform them when to divide, when to differentiate, when and where to move and, if required, when to commit suicide. These signals, to be understood properly, require the cell to express a specific selection of genes. Key proteins implicated in these functions have been strongly associated with the formation of cancer. Specifically, some "tumour suppressors" may be inhibited by mutations and other "proto-oncogenes" may be activated. Typically, a series of several mutations in these genes is required before a normal cell will transform into a cancer cell (12).

Cancers develop progressively, with tumours demonstrating different levels of abnormality along the way, from being benign to metastatic. Biochemical and genetic markers were used to determine that human tumours are monoclonal (descended from one ancestral cell) rather than polyclonal (descended from different subpopulations of cells) (13). Furthermore, the

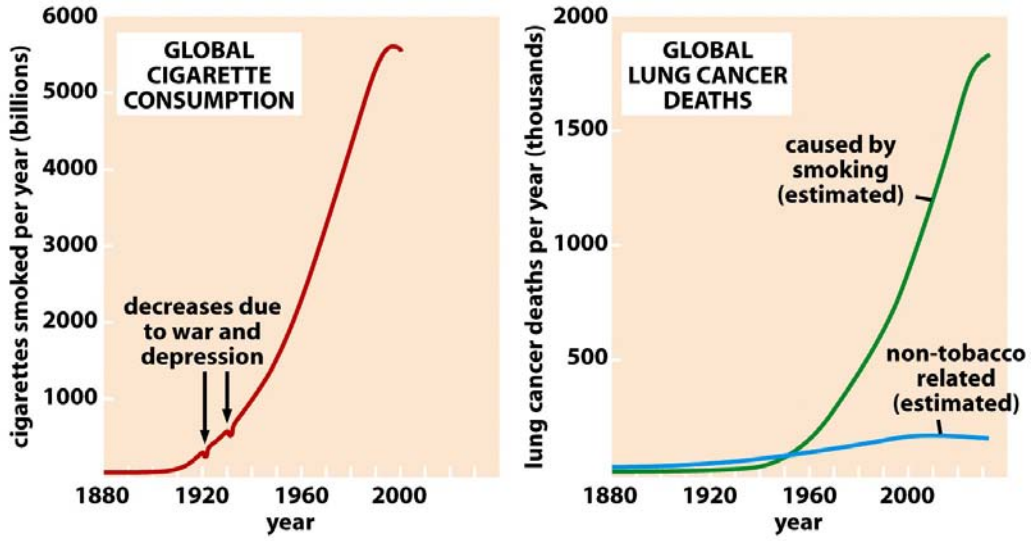


Figure1: Cigarette consumption and lung cancer. These curves compare the annual global consumption of cigarettes (billions smoked per annum, red curve, left panel) with the recorded and predicted annual worldwide mortality from tobacco-induced lung cancer is estimated to peak sometime in the fourth or fifth decade of the twenty-first century, there was an increase in what is judged to be "non-tobacco-related" lung cancer mortality (blue curve). *The Biology of Cancer* (© Garland Science 2007).

initiator cell is now believed to be a stem cell, termed cancer stem cell (CSC), that has lost the regulation growth controls but has retained its self-renewing capacity (14). This accumulation of mutations leading to cancer could be a long process. Here are some important families of genes that are likely to play a role in the development of cancer. The first family is DNA repair machinery genes. Indeed, if a cell loses its ability to repair its DNA, mutations will accumulate more easily. The second group are apoptosis genes. This group is related to the first family since irreparable mutations in DNA would normally trigger cell suicide (apoptosis). The third group is genes implicated in mortality. Cells will usually stop dividing after time, a process called senescence. A lost capacity for senescence leads to limitless replicative potential (immortality) (15). The fourth group is genes involved in sensitivity for growth signal. The fifth are genes

involved in angiogenesis, which help sustain a supply of nutrients to the tumour. The sixth are genes involved in invasion and establishment into neighbouring tissues (metastasis) (12).

It may appear unlikely that all of these gene functions must be altered by mutations to cause cancer. One should keep in mind that an adult human being has approximately 10^{13} cells. With continuous cell replacement through cell division, the aggregate number of cells that are formed during an average human lifetime is about 10^{16} cells. If we take in account that some mutations will be transmitted to the progenitor cells, the appearance of cancer is more plausible. It also partially explains how the risk of cancer increases with age. In addition, mutation in one gene could potentially affect several groups of require events. This is the case for the gene coding for the P53 protein that causes genomic instability, evasion of apoptosis and increased angiogenesis (16).

Although these subjects are of great importance for the general understanding of cancer and disease progression, the focus of the remaining literature review is on cancer chemotherapy and drug resistance.

Cancer Treatment

Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods. The choice of therapy depends on the location and grade of the tumour and the stage of the disease, as well as the general health of the patient. A number of experimental cancer treatments are constantly being developed.

Complete removal of the cancer without damage to the rest of the body is the goal of cancer treatment. Sometimes this can be accomplished by surgery, but the tendency of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body, while radiation also cause damage to normal tissue.

The combinations of mutations observed in the clinic will vary, which has lead to the observation that all cancers are unique. This fact is an

impediment to find an ideal way to treat the disease. With the genotyping tools now available, a more realistic approach could be to genetically determine each individual cancer and then deliver a specific appropriate treatment.

Chemotherapy

As with many important discoveries, the first antineoplastic drug was found accidentally. During the First World War, sulphur mustard gas was used in chemical warfare. Autopsies of soldiers exposed to this gas showed that they had profound lymphoid hypoplasia and myelossupression. This observation led the thoracic surgeon Gustav Lindskog, as encouraged by others scientists, to inject nitrogen mustard into the bloodstream of a patient with advanced non-Hodgkin's lymphoma (17). The mediastinal and lymphatic masses of the patient regressed. The remission was transient but the principle of chemotherapy was born. The same scientists pursued these studies and showed that the mustard compound behaved as an alkylating agent on DNA purine bases. This DNA damaging agent was found to be effective at any point of the cell cycle, which thereby promoted apoptosis. Since then, several other compounds have been developed with different mechanisms of action.

Anticancer drugs take advantage of characteristics that are specific to tumour cells, primarily their high proliferation rate. Certain drugs affect DNA synthesis such as methotrexate and cytosine arabinoside. These are effective during mitosis and target rapidly dividing cells such as cancer cells. There are additional compounds that affect tubulin polymerization such as paclitaxel (taxol), which stimulates tubulin polymerization, and the *Vinca* alkaloids such as vincristine, which inhibits tubulin polymerization (18,19). The resulting arrested state of mitosis induced by these drugs leads to the induction of cell death. In addition, a variety of topoisomerase I and II inhibitors have been used as antineoplastic drugs. Topoisomerases interact with DNA and are important in replication, transcription and repair. Topoisomerase inhibitors include drugs such as camptothecin and its derivatives, etoposide and teniposide. Other drugs target vascularization factors or hormone metabolism (20).

Since its introduction, chemotherapy has helped to extend life and cure millions of people. The compounds are now more specific and less aggressive, although a major problem persists: drug resistance.

Drug resistance

Drug resistance is the major cause of treatment failure in clinical oncology (21). Some tumours may be intrinsically resistant to chemotherapy prior the treatment while others, initially sensitive to chemotherapy, can acquire resistance during treatment. Furthermore, MDR may develop; this is a process that involves cross-resistance to a range of chemically unrelated agents with different cellular targets. Several mechanisms can cause MDR; for example, decreased influx of drugs or increased drug efflux mediated by membrane transport proteins can cause this phenotype (22). Moreover, the overexpression of anti-apoptotic proteins Bcl-2 and survivin or mutations in the p53 protein decreases the apoptotic potential of a cell, thus promoting MDR (23,24). Increased expression of DNA repair and glutathione-associated enzymes are other examples of mechanisms that can lead to MDR (25,26). However, one of the most intensively studied mechanisms involves the overexpression of specific ABC proteins that mediate active drug efflux (21). Indeed, the expression of these proteins decreases the accumulation of the active toxic compounds, thereby reducing their toxicity. Classically, MDR induced by ABC transporter expression is seen as acquired resistance at the tumour level. With the recent resurgence of the CSC hypothesis, it appears that the view of MDR will change again. Indeed, the cancer initiator cells with stem cell properties may overexpress ABC transporters (27,28). Thus, those few stem cells would intrinsically be MDR and the cause of relapses (see figure 2).

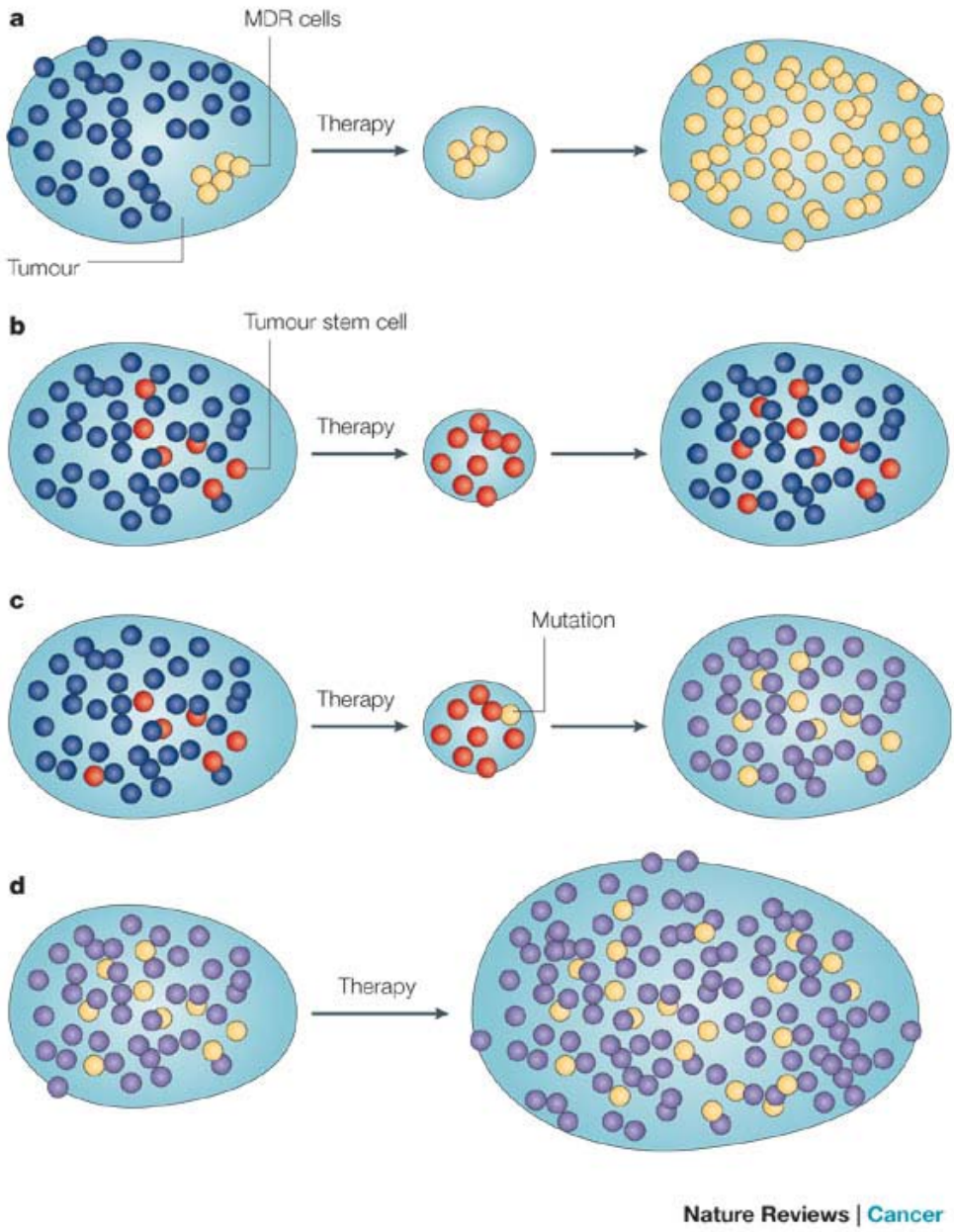


Figure 2: Cancer stem cells and drug resistance. a) In the conventional model of tumour-cell drug resistance, rare cells with genetic alterations that confer multidrug resistance (MDR) form a drug-resistant clone (yellow). Following chemotherapy, these cells survive and proliferate, forming a recurrent tumour that is composed of offspring of the drug-resistant clone. b) In the cancer-stem-cell model, drug resistance can be mediated by stem cells. In this model, tumours contain a small population of tumour stem cells (red) and

their differentiated offspring that are committed to a particular lineage (blue). Following chemotherapy, the committed cells are killed, but the stem cells, which express drug transporters, survive. These cells repopulate the tumour, resulting in a heterogeneous tumour composed of stem cells and committed but variably differentiated offspring. c) In the 'acquired resistance' stem-cell model, the tumour stem cells (red), which express drug transporters, survive the therapy, whereas the committed but variably differentiated cells are killed. Mutation(s) in the surviving tumour stem cells (yellow) and their descendants (purple) can arise (by mechanisms such as point mutations, gene activation or gene amplification), conferring a drug-resistant phenotype. As in model a, the stem cell with the aquired mutations could be present in the population before therapy. d) In the 'intrinsic resistance' model, both the stem cells (yellow) and the variably differentiated cells (purple) are inherently drug resistant, so therapies have little or no effect, resulting in tumour growth (28).

ABC Transporters

ABC transporters generality

The first of three human ABC transporters discovered to contribute significantly to clinical drug resistance was the 170 kDa P-glycoprotein (MDR1/ABCB1). Increased expression of this protein was associated with the acquisition of MDR in drug selected tumour cell lines (1,29). In 1987, a different form of MDR was described in doxorubicin selected resistant human tumour cell lines and referred to as non-P-glycoprotein-mediated MDR (30,31). The second ABC drug transporter, multidrug resistance-associated protein (MRP1/ABCC1), was cloned later from a small cell lung cancer (SCLC) cell line (H69/AR) (4). Finally, the breast cancer resistance protein (BCRP/ABCG2) was cloned in 1998 from a drug selected breast cancer cell line (32,33). The significance of each of these transporters in clinical drug resistance is still being investigated.

With the sequencing of the human genome, it became clear that there were 48 human ABC transporters subdivided in 7 families from A to G (34,35). However, further studies have suggested that many ABC transporters have a restricted substrate profile, and to date only 13 have been shown to transport chemotherapeutic drugs. Only three multidrug resistance 1/P-glycoprotein 1 (MDR-1/P-gp1 or ABCB1), multidrug resistance-associated protein 1 (MRP1 or ABCC1) and the breast cancer resistance protein (BCRP or ABCG2), play a major role in the normal physiology of the cell as well as drug resistance (36). Many ABC transporter genes have been knocked out in the mice model including P-gp and MRP1, with no effect on animal survival (37). ABCC-2 to 5 have also been suggested to have clinical significance in drug resistance, but need further investigation (38).

ABC transporters share a common architecture and a common basic mechanism: two ABC or nucleotide binding domains (NBDs) and hydrolyze

ATP to provide energy for unidirectional substrate transport. This occurs through a translocational pathway provided by two membrane spanning domains (MSDs) each containing 6 transmembrane (TM) α-helices (39). As seen in figure 3, this general structure varies slightly, as some ABCC family proteins possess a third MSD, while ABCG2 acts as a half transporter requiring the formation of a homodimer to be active (40). The NBDs, but not the TMDs, are homologous throughout the family and have several characteristic motifs including the Walker A and B motifs common to many nucleotide binding proteins, and others such as the ABC signature motif (C), and staking aromatic D, H and Q loops, which are unique to the ABC transporters (41-43). By contrast, the MSDs only exhibit significant sequence similarity if they transport chemically related substrates in the same direction across the membrane (44).

As seen in figure 3, ABC transporters have a wide variety of non related substrates that are both endogenous and exogenous. This fact suggests that the substrates will not bind in a traditional "lock and key" fashion. Instead, they are more likely to fit in a hydrophobic pocket which could interact with different affinities depending on the various substrates. Nonetheless, some binding sites or regions are identified as being important. The use of various substrates and various P-gp sources lead to the determination of TM segments important for binding (45). As we see in the schematic representation of the topological structure of P-gp in figure 3 and 5, these key TM segments have been shown to include TM 4 to 6 and 10 to 12. In MRP1 the use of GSH and LTC_4 radio-labelled with photoreactive analogs enabled the elucidation of TMs 10 to 12 and 16 and 17 as being involved in drug binding (46,47). Interestingly, if the extra MSD of MRP1 made of 5 TM segments is added, the regions of P-gp and MRP1 overlap. The MSD0 of MRP1 is also photolabelled with an LTC_4 analog as well as the two cytoplasmic loops by the GSH analog. Besides the specific MSD0 characteristic of MRP1, the similarity between MRP1 and P-gp supports a common structure and mechanism for the ABC transporters.

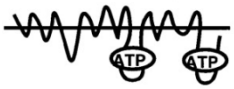
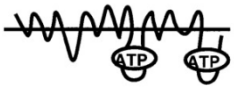
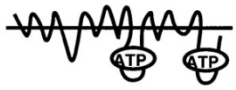
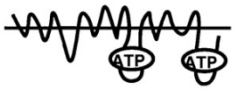
Common Names	Official Name	Structure	Substrates	Normal location
P-gp, MDR1	ABC B1		Neutral and cationic organic compounds	intestine, liver, kidney, blood-brain barrier
MRP1	ABC C1		GS-X and other conjugates, organic anions	widespread
MRP2, cMOAT	ABC C2		GS-X and other conjugates, organic anions	liver, kidney, intestine
MRP3, MOAT-D	ABC C3		GS-X conjugates, anti-folates, bile acids, etoposide,	pancreas, kidney, intestine, liver, adrenal
MRP4, MOAT-B	ABC C4		nucleoside analogs, methotrexate	prostate, testis, ovary, intestine, pancreas, lung
MRP5, MOAT-C	ABC C5		nucleoside analogs, cyclic nucleotides, organic anions	widespread
MRP6, MOAT-E	ABC C6		anionic cyclic pentapeptide	liver, kidney
MXR, BCRP, ABC-P	ABC G2		anthracyclines, mitoxantrone	placenta, intestine, breast, liver

Figure 3. ABC transporters with known drug substrates. Curved lines represent transmembrane domains, and the ATP in the ovals represents the ATP-binding cassettes (45).

Crystal structures of some bacterial ABC transporters were recently characterized; Sav1866, BtuCD, HI1470/1 from *S. aureaus*, *E. coli* and *H. influenzae,* respectively (48-50). With these structures and the extensive biochemical characterisation of P-gp, several mechanisms of action have been proposed for drug extrusion by an MDR exporter. The model presented here (the ATP switch model) is the most recent and continues to be consistent with the ongoing findings (51). Briefly, as seen in figure 4 the mechanism is completed in four steps. Step 1: the ligand binds to the TMDs in the high-affinity open NBD dimer conformation, inducing increased affinity for ATP. Step 2: ATP binding induces formation of the closed NBD dimer, which in turn induces a large conformational change in the TMDs sufficient to translocate the

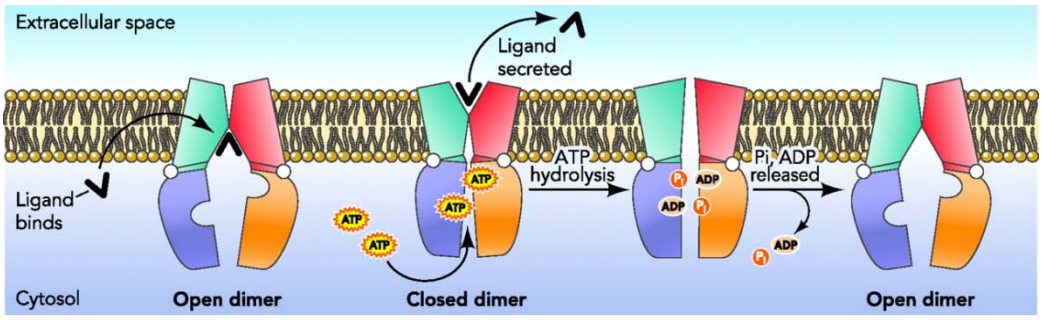


Figure 4 ATP-switch mechanism of ABC transporter (43).

ligand. Step 3: ATP hydrolysis initiates dissolution of the closed NBD dimmer and finally step 4: P_i, then ADP are released to complete the transport cycle and restore the protein to a high-affinity state for the next ligand to bind and be translocated.

General structures of ABC transporters is helping to elucidate the mechanisms of action of ABC transporters, although, complete understanding is far from being reached. These proteins seem to depend significantly on their environment for their activity, something that is often not taken into account in crystallography. Relations between environment and activity will be discussin the P-glycoprotein section below.

P-Glycoprotein

ABCB1 was the first identified mammalian ABC transporter. Its discovery result from a stepwise selection of colchicine resistant clones from the Chinese hamster ovary cells, AuxB1. These clones were called Ch^R (52). Soon after this initial observation, it as been noticed a 165 kDa glycosylated protein exclusively in the Ch^R clones (1). Since these cells had increased colchicine membrane *permeability*, the protein was called: P-glycoprotein. In 1979, the protein was finally purified and was shown to be expressed at the plasma membrane (53). It is interesting to note here that the protein was first resolved by SDS-PAGE using a Fairbanks system (containing urea and avoiding sample boiling (54)), which at the time was favoured by membrane biologists. This was important since the use of the standard Laemmli system

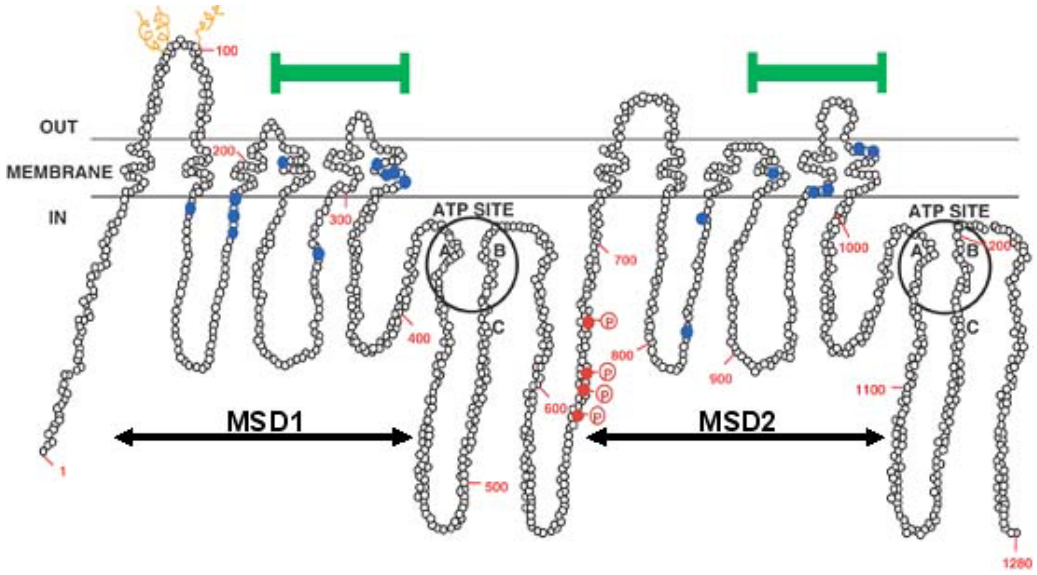


Figure 5. A hypothetical 2-D model of human P-glycoprotein. Based on hydropathy analysis of the amino acid sequence and its functional domains. In this diagram, each circle represents an amino acid residue with blue solid circles showing the positions of some mutations that alter the substrate specificity of P-gp. The MSDs regions are indicated with double headed arrows. The ATP sites (NBDs) are circled, with Walker A, B and the signature or C region indicated as A, B, and C, respectively. The N-linked glycosylation sites (N91, N94, and N99) are indicated by squiggly lines in the first extracellular loop, and the phosphorylation sites (S661, S667, S671, and S683) in the linker region are shown as a red-circled P. The green bars above the TM domains 4–6 and 10–12 show the regions labeled with photoaffinity analogues. Modified from (45).

(55) greatly reduced sensitivity for P-glycoprotein detection (56). In 1986, it was demonstrated that the overexpression in Ch^R lines was correlatedwith the amplification of the *mdr1* gene when pulse field experiments showed that the amplicon contained 5 genes (57). In 1986, the direct relation was made when the gene encoding mouse P-gp was cloned, and its expression was shown to induce MDR (58). Shortly after, the same correlation was made with the human

gene (59). The generation of a monoclonal antibody specific for human P-gp, MRK16 (60), led to the first papers describing patterns of human P-gp expression in the apical portion of intestinal, liver, and kidney epithelia (61,62).

More recently, P-gp was found to be expressed in stem cells and in what is now believed to be the tumour initiator cells, so-called cancer stem cells (63,64). Because of its localization and its role in drug resistance, normal P-gp function is now associated with cellular protection against xenobiotics. Although, the "P" in P-glycoprotein stands for permeability, it is now clear that P-gp expression leads to drug resistance through a drug efflux. This mechanism implies that initial binding of the substrate to P-gp is required. To demonstrate this, photoaffinity labelling experiments were used to elucidate this theory (65). The same technique was also used to demonstrate the binding of ATP to P-gp (66). The suggestion of an ATP dependant mechanism for drug efflux by P-gp came when inside out plasma membrane vesicles containing P-gp were isolated and shown to accumulate MDR compounds in the presence of ATP (67). The confirmation of ATPase activity by P-gp was made not long after (68). Biochemical characterization of both human and hamster purified P-gps has determined that both ATP binding sites are capable of hydrolysing ATP but not simultaneously, and the stoichiometry of ATP hydrolysis is 1 mol ATP/mol of P-gp (69-71). Furthermore, ATP hydrolysis and drug transport are obligatorily linked (72). The mutations (depicted as blue filled circles in figure 5) in mammalian P-gps that affect substrate specificity are found throughout the molecule, including the TM regions, the soluble intra- and extracellular loops, and the ATP-binding/utilization domains (29). Also, P-gp is glycosylated at three sites (N91, N94, and N99) in the first extracellular loop (wiggly lines in the first extracellular loop in Figure 5) (73). The glycosylation appears to be required for the proper trafficking of the transporter to the cell surface, but it is not required for the transport function of P-gp (73,74). P-gp is also a potent substrate for phosphorylation by protein kinase A and C at four serine residues in the linker region (S661, S667, S671, and S683, denoted as a red circled P in

Figure 5). However, a P-gp mutant lacking all phosphorylation sites exhibits normal transport function, and confers drug resistance to sensitive cells (75).

The discovery of P-gp has launched an important new field of research mainly because of the possibility of being a key to the resolution of cancer MDR. As is often the case, the problem was more complex than initially thought and more than 40 years later attempts to resolve it remained elusive. Although, recent years have shed light on unexpected aspects of the protein and there is hope again.

P-gp ATPase

P-gp hydrolyses ATP in the absence of known substrates when reconstituted in a lipid bilayer environment; this is defined as the basal ATPase activity. The ATPase activity is significantly enhanced in the presence of many transported substrates and so-called modulators. The latter activate the P-gp ATPase activity without undergoing a significant P-gp related net transport. This phenomenon may occur for compounds that are translocated by P-gp but have a high passive permeation rate across the membrane, exceeding the transport efficiency of the protein (76).

It has been reported that cholesterol stimulates basal (i.e. without any drugs) ATPase activity (77,78), and that cholesterol is recognized and transported by MDR1 (79). It was also shown that the depletion of cholesterol reduced the transport activity of MDR1, resulting in the intracellular accumulation of drugs in cells (78,80,81). Due to these properties, cholesterol can be judged as a P-gp modulator. Many other modulators have been characterized by Stein and Orlowski groups (82,83). The two that were most extensively studied are the calcium channel blocker verapamil (Vrp) and the steroid hormone progesterone (Pro). These were the first two P-gp inhibitors identified, and are sometime still used as such (9). Regarding these two modulators, studies have suggested that they do not compete for the same binding sites and that they would cooperate in activating P-gp ATPase (8,9). Using proteoliposomes, the same cooperation was also seen for cholesterol

regarding these two modulators for cholesterol (84). Moreover, when no cholesterol was added to the proteoliposomes the stimulation was totally abolished as well as the basal ATPase activity. Therefore, it appears that cholesterol could be a substrate for P-gp but also, P-gp is more active when the membrane fluidity is diminished by the presence of cholesterol (77).

P-gp in Clinical Studies

In 1985, P-gp was detected in clinical samples (85). Another important study using 1000 human cancers, where MDR1 mRNA expression was assessed, lead to further important clinical findings (86). It showed that MDR1 was expressed at levels judged to be sufficient to confer MDR in many epithelial cancers derived from tissues that were known to express MDR1 (colon, liver and kidney) but also in hematopoietic cancers [acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and lymphoma)] as well as in solid tumours (breast and ovary). It is interesting to note that the majority of the tissues with P-gp mRNA levels judged sufficient to lead to MDR led relapse following chemotherapy with known P-gp substrates. Another important discovery correlates MDR1 gene expression and clinical MDR. Mickley et al. demonstrated that rearrangements involving an important upstream promoter of MDR1 frequently occur in drug-resistant cancers (87). In 2001, a clinical trial showed a survival advantage for patients with AML treated with MDR inhibition plus chemotherapy (88). Finally, since P-gp seems to be expressed in tumour initiator cells, it has been suggested that these few important cells will invariably show MDR, explaining the occurrence relapses (27).

The idea of reversing MDR has been present since MDR first appeared, but the demonstration in 1981 that verapamil could reverse MDR gave meaningto this idea (89), even though the calcium channel activity of verapamil was too toxic to pursue in clinical trials. This was a first generation inhibitor such as the immunosuppressant cyclosporin (90). A cyclosporin analog, PSC833, has been a promising second generation inhibitor, but it also failed in

clinical trials. Nontheless it remains a useful laboratory tool. Finally, a third generation of compounds have also failed to significantly improve patient survival significantly after phase three clinical trials (91).

Blocking P-gp as a strategy to reverse MDR has yet to give encouraging results, therefore alternative ideas are needed. One interesting approach is the exploitation of the paradoxal collateral sensitivity feature that elicits some MDR cells.

P-gp collateral sensitivity

Collateral sensitivity is defined as the hypersensitivity of a cell line to a certain compound while showing resistance to others. In P-gp expressing cells, collateral sensitivity to the glycolysis inhibitor 2-Deoxyglucose, to the extrinsic apoptosis pathway inducer TRAIL and to NSC73306 (a thiose-micarbazone), was observed (92-94). The first paper report of collateral sensitivity arrived almost at the same time as the first MDR cell lines were selected, and by the same group. Indeed, in 1976, Victor Ling's group showed that their colchicine-resistant CHO cells were hypersensitive to steroid hormones and detergents (2). Since then, the phenomenon has been reported numerous times but never fully understood.

The most studied case of collateral sensitivity occurs with calcium channel blockers and MDR selected CHO cell lines. In 1986, Warr et al. reported collateral sensitivity to verapamil in selected CHO cell lines that are resistant to vincristine (5). The same article also provides important information about the Ca^{2+} status in these cells; first it demonstrated that a small but significant accumulation of calcium was stimulated by the addition of active concentrations (leading to collateral sensitivity) of verapamil in both the collaterally sensitive and their parental counterpart, and second, the total calcium content of the collaterally sensitive cells was higher relative to their parental. In this paper, they also underlined the fact that not all CHO cells selected for resistance to antimicrotubular drugs were verapamil hypersensitive, thereby predicting that resolution of the mechanism would not be

straightforward. Since no reduction of Ca^{2+} intake by verapamil in both the parental and the collaterally sensitive cells was observed, they judged that the action of verapamil on calcium channels was not related to the hypersensitivity in P-gp expressing cells. A year latter, another group repeated the Ca^{2+} entry inhibition experiments with verapamil, this time using another selection of CHO cells. Their results showed no inhibition (6). This finding, which was not shown in the first article, was explained by the possibility that these cells do not have functional voltage dependant calcium channels. This second paper sealed the dogma that the action of blocking calcium channels is not related to collateral sensitivity. The same year, transfection in mouse fibroblast cells (NIH 3T3) with the mouse *mdr* gene also lead to verapamil collateral sensitivity (95). This showed the phenomenon to be non specific for hamster P-gp or hamster cells.

A year later Warr et al. showed other calcium channel blockers (diltiazem and nicardipine) and a "membrane active compound" (quinidine sulphate) to induce collateral sensitivity (96). In this report they showed the over-expression of low molecular weight proteins in the collaterally sensitive cells. Growth of these cells during a prolonged absence of selecting agent leads to the reversing of both MDR and collateral sensitivity. In these revertants, the expression of the low molecular weight proteins returned to the parental level. Those proteins were suggested to be two post-translational products of the calcium binding protein sorcin; this 22 kDa protein was already demonstrated to be expressed in MDR cells (57).

Conversely, the correlation between P-gp and/or sorcin expression and collateral sensitivity was found to be the opposite in another study. Reeve et al. showed a correlation between the expression of P-gp and sorcin, but in addition to being resistant to the usual compounds, these cells were also proportionally resistant to verapamil (97). In 1991, Warr's group showed that there was a correlation between P-gp gene amplification, mRNA and surface protein expression, increased P-gp expression with the collateral sensitivity level (98). They also investigated the four others genes found in the same amplicon and

found that their amplification was much lower. In addition, sorcin amplification and mRNA expression did not correlate with the collaterals sensitivity level. In 1995, Cano-Gauci et al. selected collaterally sensitive cells for resistance to verapamil (99). These cells kept their P-gp expression profile as well as their MDR phenotypes. The latter study suggests that if P-gp is important for the collateral sensitivity phenomenon, it is not the only factor. Finally, in 2003 aided by new techniques, Karwatsky et al. showed that the collateral sensitivity to verapamil was induced through apoptosis (7). They also correlated the P-gp ATP consumption activity with the phenomenon and then suggested that the ensuing stimulated oxidative phosphorylation was leading to accumulation of toxic ROS, see Figure 6.

Overall, the mechanism of collateral sensitivity to Vrp was only detected in hamster and mouse models and seems to be correlated with P-gp expression. There is a possibility for a second factor since selection for resistance to vrp while maintaining P-gp level is possible. Higher levels of Ca2+ can be detected in some collaterally sensitive cells, a phenomenon that could be attributed to higher sorcin expression. Vrp does not seem to block calcium channels in these cells, on the contrary, it seems to increase Ca2+ levels in the cells. Finally, the Vrp stimulation of P-gp ATPase activity correlates with apoptosis cell death.

Multidrug resistant-associated protein 1

ABCC1 was first isolated from a doxorubicin-resistant small cell lung cancer line named H69AR (100). The protein consists of 1531 amino acids, and when fully glycosylated, has a molecular mass of 190 kDa. ABCC1 is expressed in most tissues of the body, and expressed at higher levels in the lungs, kidney, testis and blood mononuclear cells (4,101). Its presence in lung cells is consistent with the proposed role for ABCC1 in protecting lung tissues from airborne xenobiotics. In polarized epithelial cells, ABCC1 is found in basolateral membranes rather than apical membranes where other transporters

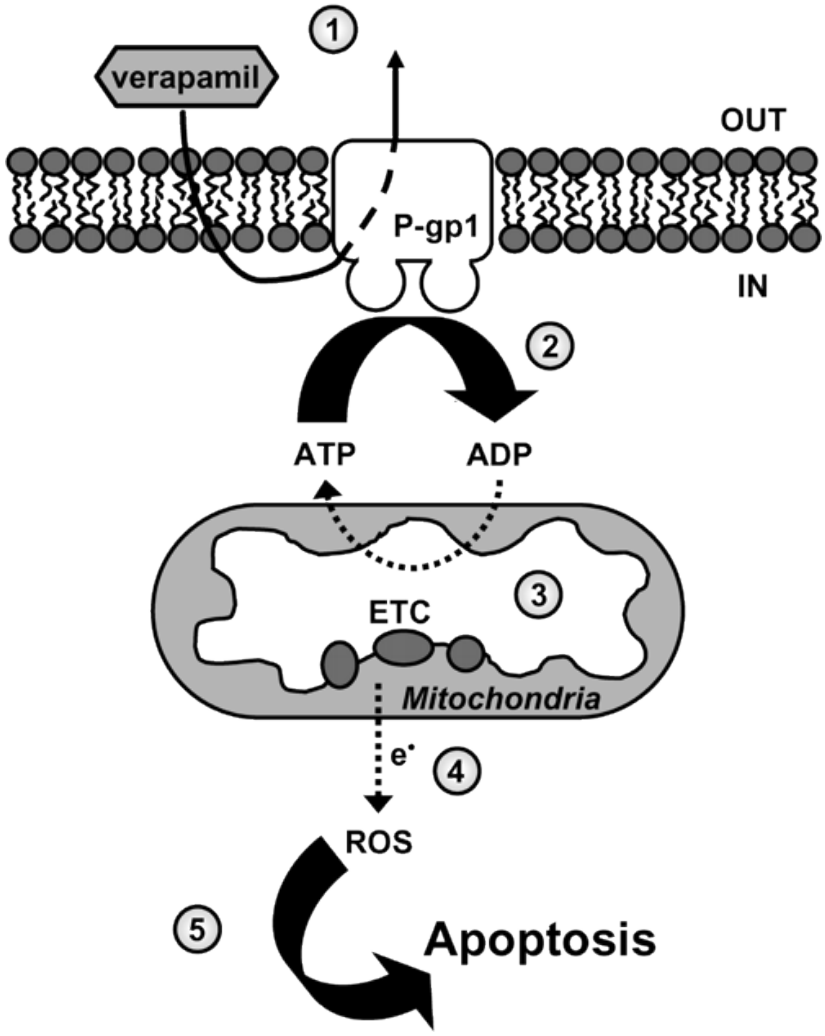


Figure 6. **Proposed mechanism of verapamil collateral sensitivity in P-gp1 expressing cells**. Verapamil crosses the cellular membrane and interacts with P-gp1, which transports verapamil back into the extracellular environment (1). The interaction with verapamil causes elevated levels of ATP hydrolysis by P-gp1 (2). This creates a high demand for ATP which is generated from oxidative phosphorylation in the mitochondria (3). As a result of the high ATP demand, electrons are lost from the electron transport chain (ETC), causing the production of higher than normal levels of ROS (4). The high concentration of

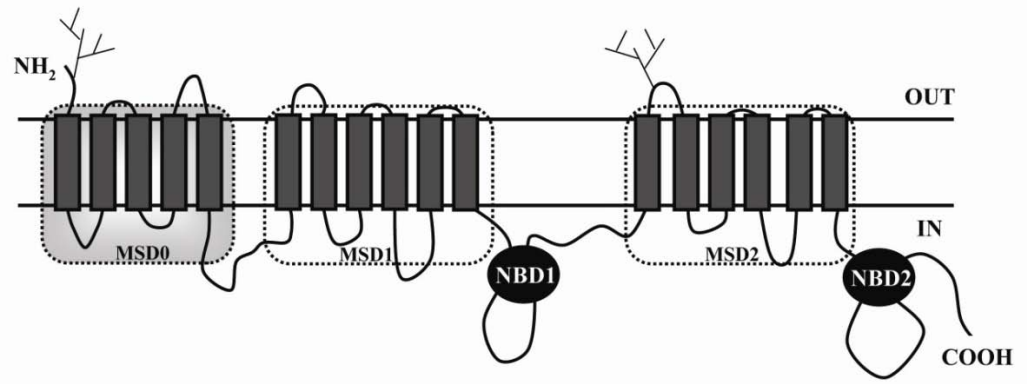


Figure 7: Topology of MRP1. Abbreviations: MSD: membrane spanning domain; NBD: nucleotide binding domain. *modified from (102).*

such as P-glycoprotein 1, ABCC2 and BCRP/ABCG2 are located. As shown in figure 7, the current models regarding the topology of ABCC1 predicts a ROS causes apoptosis by damaging lipids and DNA, or by initiating the cytocrome c apoptotic pathway (5) (7).secondary structure with 17 TM helices distributed in three membrane spanning domains (MSD) (103). The first two MSDs (MSD0 and MSD1) encode 11 TM domains. MRP1 also encodes two nucleotide binding domains NBDs (NBD1 and NBD2), that follow MSD1 and MSD2, respectively (40). Of the potential N-linked glycosylation sites in MRP1, only three are utilized in mammalian cells (104), and studies to date indicate that glycosylation is not essential for MRP1 transport function (105,106). Despite the similarities in their secondary structure, P-gp1 and ABCC1 share different drug specificity profiles, in part due to the additional N-terminal extension of ABCC1 (107). ABCC1 has a broad substrate specificity that includes: natural products, non-anionic anticancer drugs, GSH, GSSG and conjugated compounds (108-111). Many of these ABCC1 substrates are either conjugated to glucoronide, sulfate or GSH, or co-transported with GSH. The glutathione transport feature of ABCC1 will further be described in detail below.

Clinical significance

The importance of ABCC1 in terms of cancer relapse is not clear, but the expression of this protein in tumours remains an indication of poor

prognosis (112). Clinical studies using flow cytometry, immunohistochemistry and q-PCR have demonstrated the presence of ABCC1 in different types of cancers, and specifically, ABCC1 overexpression is shown in acute myelocytic leukemic cells (AML), breast cancer, sarcoma, bladder cancer, central nervous system cancer and lung cancer (36). In lung cancer, using q-PCR, high levels of ABCC1 mRNA expression was shown in all tumours from patients with non-small cell lung cancer (NSCLC), and in about ninety percent of tumours from small cell lung cancer (SCLC) patients (113,114). Together, these results support a strong association between lung cancer patients and ABCC1 expression levels, and places an urgent need on the understanding of ABCC1s role in drug transport and its contribution to MDR.

Collateral sensitivity

In 1989, Dr. Cole found the overexpression of ABCC1 in SCLC H69, cells that had been selected with doxorubicin; these MDR cells were termed H69AR. At this time, only P-gp1 had been shown to cause MDR. In addition to the MDR phenotype, the cells overexpressing ABCC1 were also found to have a reduction in intracellular GSH levels. After further investigating the activity levels of enzymes implicated in GSH metabolism, results pointed to an increase in intracellular levels of GSH rather than the expected decreasing GSH levels. This result implied that another mechanism in the cells was influencing GSH levels other than GSH-metabolizing enzymes. After treating H69AR cells with the GSH synthesis inhibitor BSO, the first example of collateral sensitivity was observed with ABCC1 (3). In addition to BSO, Vrp has been observed to cause collateral sensitivity in ABCC1 expressing cells (115,116). Vrp is an ABCC1-mediated GSH extrusion enhancer. The collateral sensitivity of ABCC1 expressing cells to both BSO and Vrp correlates with the ability of each compound to reduce intracellular GSH levels.

Modulation of GSH levels in MRP1 expressing cells

L-S,R-buthionine sulfoximine

BSO is an analog of methionine sulfoximine (MSO), which was identified as the toxic agent in agenized grain BSO is the most specific and least toxic inhibitor of γ-GCS, causing GSH depletion in cells (117). Enzyme inhibition of γ-GCS follows pseudo-first order kinetics. BSO binding to γ-GCS is non-covalent and apparently non reversible in the presence of MgATP (117). *In vivo* studies using BSO in mice have shown that intra-peritoneal (i.p.) admistration favours GSH depletion in plasma, liver, and kidney, while oral administration was shown to give better results in lung and stomach with considerably less drug depletion in the liver (118). In mice, xenograft studies, using three different tumour cell lines, demonstrated that GSH depletion could be achieved in tumours with doses of BSO lower than those required for normal tissues (119). *In vitro*, Zaman et al. have previously demonstrated that depletion of GSH by BSO led to the complete reversal of resistance to doxorubicin, daunorubicin, vincristine and VP-16 in lung carcinoma cells tranfected with ABCC1 (120). This effect has been further confirmed *in vivo* with xenografts of ABCC1 expressing doxorubicin-resistant cells in mice. After BSO treatment, the response to doxorubicin in mice was completely restored without potentiating host toxicity (121). Clinical trials using BSO combined with melphalan (L-PAM) show that a 40 % reduction in GSH levels in peripheral cells is well tolerated (122). Together, it has been determined that treatment with BSO induces cell death caused by depletion of GSH (123,124). Given these studies, BSO may be used in clinical oncology.

Apigenin

Api is one of the most common flavanoids found in many fruits and vegetables. Api has been shown to possess anti-inflammatory, anti-carcinogenic and free radical scavenging properties in many *in vitro* systems (125). It is a relatively non-toxic, non-mutagenic flavone subclass molecule and one of the most promising flavanoids with chemopreventive activity (126). Api has been shown to be a potent inhibitor of NF-kB transcription, and has been shown to

decrease Bcl-2 protein levels and increase the p53 expression causing cell cycle arrest and apoptosis in several tumour cell lines (127-129). Direct molecular targets of Api may include heat shock proteins (130), telomerase (131), ornithine decarboxylase (132) and the ATPase domain of kinases (133,134). *In vivo*, 25 mg/kg of Api i.p. administrered during a 7 day period resulted in a 50 % reduction of induced neuroblastoma tumour mass in rats without side effects (morbidity, weight lost, organ abnormalities) (128). This drug quantity has been shown to give a sera concentration maximum of 31 μM (135). Another study performed in mice gave similar results with the B16-BL6 melanoma cell line. In that study, an 85 % reduction in tumour cell invasion was also observed with Api (136). This anti-invasiveness property of Api has further been demonstrated *in vitro* when connexin43 (an invasiveness promoter) transfected HeLa cells lost their motility after treatment with Api (137). Interestingly, Api was shown to stimulate GSH transport by ABCC1 (138).

Verapamil

Vrp is a well known calcium channel blocker used in clinical settings for its antihypertensive properties (139). At the concentration used, up to 500 mg daily, there are only minimal side effects (140). This compound was shown to reverse P-gp1-mediated MDR (89,141). Unfortunately, high plasma levels are required to modulate P-gp1 efflux mechanisms. Using four times the amount needed for treating hypertension in patients with P-gp1 expressing tumours, this treatment regiment only partially restored the response to drug treatment. The Vrp concentration in the blood of these patients was as high as 1.342 μg/ml (2.73 μM). These plasma levels are associated with major side effects, including non life-threatening hypotension and cardiac arrhythmia (142). Data from the literature on human poisoning provided a lethal blood concentration of 13 μg/ml (55 μM) (143). Depending on the concentration, Vrp has different effects on P-gp1. At high concentrations, it is an inhibitor of this ATP-dependant transporter; at low concentrations, it stimulates ATP hydrolysis activity (82,144). It has also been demonstrated that P-gp1 overexpressing

cancer cells show collateral sensitivity to Vrp mediated by apoptosis (7). This is the result of ROS production from metabolism to compensate for the reduced ATP levels. Like Api, Vrp stimulates GSH extrusion by ABCC1 (145). Moreover, this increase in GSH export induces collateral sensitivity in tumour cells overexpressing ABCC1 through apoptosis (115).

Reactive Oxygen Species and Cell Death

Metabolism and ROS

In cells approximately 90% of the reactive oxygen species (ROS) are coming from the mitochondria (146). It was initially suggested that up to 2 % of the total oxygen consumption was eventually used for ROS formation (147). More data reduced this estimation to ~ 0.2 % (148,149). Discovery that superoxide dismutase (SOD) was a major step for establishing the generation of ROS in the mitochondria. There are two SOD types within the cell; SOD1, a copper-dependent cytoplasmic enzyme, and SOD2, a manganese-dependent enzyme found in the mitochondrial matrix. They both convert O_2^- into H_2O_2, which is further deactivated by catalase (150) or by glutathione peroxidase GPx. Peroxiredoxin is another mitochondrial enzyme family enzyme scavenging ROS (151).

The primary ROS generated in the mitochondria is superoxide (O_2^-). It is believe that there are at least two main sources of O_2^-. The first one would be at the complex I and the second would be at the complex III (152).

Calcium and ROS in cell death

In resting cells, the cytosolic free Ca^{2+} concentration is maintained at approximately 100 nM, but through mobilization from intracellular stores (such as the endoplasmic reticulum (ER), Golgi or lysosomes (153-155)) or entry across the plasma membrane, free Ca^{2+} can increase to >1 µM (153,156). Maintaining calcium homeostasis is important and, there are proteins with the capability of sequester the free Ca^{2+} (157). Sorcin is a sequestering protein that has initially been found in MDR cells (57). Interestingly, Ca^{2+} was demonstrated to help trigger and modulate apoptotic cell death. Indeed, increase

in Ca^{2+} was shown to stimulate both cytochrome C release and caspase activation (158). Both processes are also stimulated by ROS increases and therefore could work cooperatively (159).

Glutathione

The reduced form of glutathione (GSH) plays a central role in biochemical processes, and disturbances in GSH homeostasis are implicated in the aetiology and progression of a number of diseases such as liver dysfunction, AIDS and pulmonary fibrosis (160). The role of this tri-peptide is to maintain the thiol redox status of a cell and thus protect it against ROS, reactive metals and electrophiles (161,162).

Protective role of GSH

GSH is the major non-protein intracellular thiol source; thus it is a major participant in redox reactions and is required to maintain a reducing environment in the cell. All aerobic organisms are subject to physiological oxidant stress as a consequence of aerobic metabolism. Indeed, the production of ATP through oxidative phosphorylation in the mitochondria leads to the formation of the superoxide radical ($O_2 \cdot^-$). This free radical can lead to the formation of ROS such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH\cdot$) (163). These reactive compounds cause lipid peroxidation and can disrupt metabolic processes. GSH is the predominant defence against these toxic products of oxygen, particularly in mitochodria (164). Endogenously produced hydrogen peroxide is reduced by GSH in the presence of GSH peroxidase. As a consequence, GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by GSSG reductase at the expense of NADPH, forming a redox cycle. Glutathione is also implicated in detoxification of diverse groups of reactive metabolites, often through GSH-conjugation by glutathione S-transferases (165,166).

Glutathione metabolism

All cells synthesize GSH (and its concentration can be as high as 10 mM in liver cells) (167). GSH is a tripeptide (Gly-Cys-Glu) and its synthesis requires two ATP dependent enzymes; γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase. Inside the cell, GSH is distributed to diverse cellular compartments with the mitochondria having the largest pool. GSH is also secreted into the extracelullar space, including the blood stream and in the bile fluids (168). Within the cell, GSH exists mainly (98 %) in the thiol reduced form (GSH), but a smaller percentage is conjugated or thiol-oxidized (gluthionine or GSSG). The GSH/GSSG ratio is maintained by the NADPH dependent enzyme glutathione reductase, (see Figure 8). GSH degradation occurs exclusively in the extracellular space and only by cells expressing the membrane-bound enzyme γ-Glutamyl transpeptidase (γ-GT).

GSH transport

GSH transport out of cells is required for the maintenance of the correct blood GSH concentration of 10 μM. A higher transport activity is seen in liver cells, generating a GSH concentration in bile of up to 10 mM. There is no known transporter specific for GSH extrusion; instead, evolution has favoured the use of more general transporters to do this task (169). Recent studies indicate that some members of the ABCC protein family are responsible for GSH extrusion (170). In particular, five of the 12 members of the MRP/CFTR family appear to export GSH from cells, namely: ABCC1, 2, 4, 5 and the cystic fibrosis transmembrane conductor regulator (CFTR). Being the first to be discovered and ubiquitously expressed, MRP1 is considered the major GSH transporter, and to date the best characterized of the MRP/CFTR family. ABCC1 transports all GSH variants, including its reduced and oxidized forms, as well as many GSH-conjugates. Initial evidence for GSH transport by this protein was shown using cells overexpressing MRP1 (120). Earlier studies have demonstrated that leukotriene C_4 (LTC_4), a glutathione s-congugated molecule,

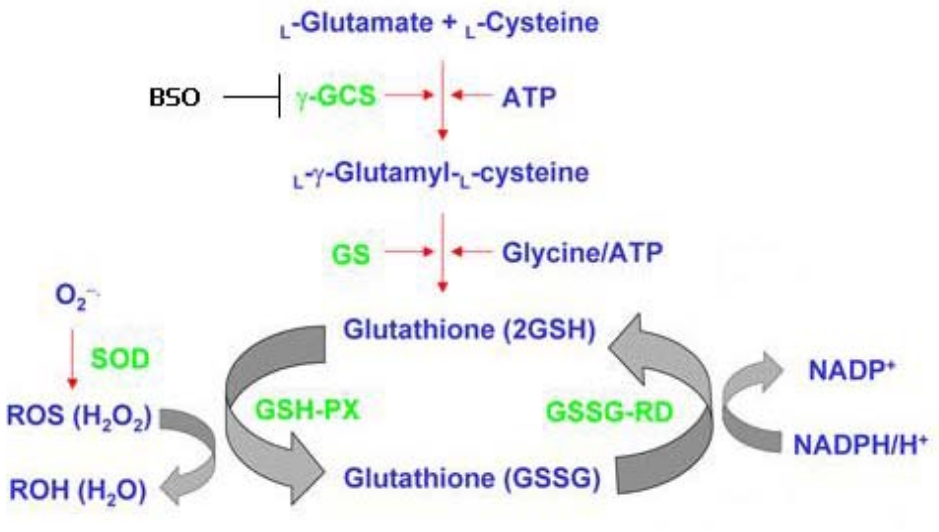


Figure 8: The schematic of the redox cycle shows the relationship between antioxidant enzymes and glutathione. Enzyme abbreviation: γ-GCS: γ-glutamylcysteine synthetase; GS: glutamyl synthase; SOD: superoxide dismutase; GSH-PX: glutathione-peroxidase; GSSG-RD: glutathione reductase. *Modified from* (171).

is also transported by ABCC1 (108,109). Indeed, this finding was the catalyst for later studies which explored the role of MRP1 in GSH transport. Considerable effort has gone into the mapping of ABCC1 drug binding site(s). Several reports by Karwatsky et al. as well as others, have mapped the binding domains of LTC4 and GSH using photo-reactive drug analogs (46,47,102,172). The affinity of GSH itself for ABCC1 appears to be quite low (K_m ~ 10-20 mM) (173); however the presence and co-transport of doxorubicin or vincristine by ABCC1 results in a dramatic increase in GSH affinity (K_m ~ 10-100 μM) for ABCC1 (120). Extrusion of other compounds could also be stimulated by the presence of GSH without the GSH itself being transported. Indeed, studies have shown that decreasing intracellular GSH levels with BSO reduces transport of daunorubicin, doxorubicin, etoposide and vincristine in cells overexpressing ABCC1 (170,174,175). Moreover, the presence of certain molecules stimulates the export of GSH without the drugs being transported by ABCC1; two such compounds that stimulate GSH transport via ABCC1 are

Vrp and apigenin (Api) (176,177). ABCC2 shares many of the characteristics of ABCC1 in terms of transport activity but its tissue expression is more restricted. Furthermore, ABCC2 also transports GSH and GSH-conjugates (170). ABCC2 is largely found in the liver, on the apical side of cell membrane (173,178,179). ABCC4 and 5 as well as CFTR protein have also been shown to promote extrusion of GSH but need further investigation. Studies using rat cells have shown that the organic anion transporting polypeptide (OATP or SLC21A) family of transporters can extrude GSH but this has yet to be confirmed in human cells (170).

Role of GSH in cell death

In addition to detoxifying cells from harmful compounds, GSH levels can modulate cell survival and death. Low GSH levels are associated with mitochondrial dysfunction, and induction of apoptosis (180,181). Furthermore, proteins involved in apoptosis also modulate cellular GSH levels. Enhanced expression of Bcl-2 or Bcl-X_L, two anti-apoptotic proteins, can increase intracellular GSH (182-185). Depletion of intracellular GSH levels in cells overexpressing Bcl-2 has been shown to result in the degradation of anti-apoptotic proteins and hypersensitivity to apoptosis (186,187). Conversely, expression of the pro-apoptotic protein p53 in p53 minus cells decreases GSH levels (188). As GSH has been found to protect against apoptosis, cells have developed a caspase stimulated GSH export system when apoptosis is initiated (189-191). In contrast, very low GSH levels have also been associated with the prevention of apoptosis (192-194). In the case of the caspases, the active sites have been shown to contain cysteines that require adequate GSH levels for proper activity (193,194). Therefore, cells with reduced GSH levels will more likely die by necrosis, apparently because of an accumulation of ROS that inhibit caspase activation (195-197). In summary, high levels of GSH prevents apoptosis, low levels of GSH causes apoptosis, while higher GSH depletion or a sudden drop leads to necrosis.

Cell Death

Different pathways lead to cell death including: autophagy, mitotic catastrophe, apoptosis and necrosis. Autophagy is a form of programmed cell death usually induced by different types of deprivation, such as growth factors and nutrient starvation; however, it may also be stimulated by a DNA damaging agents (24). Mitotic catastrophe is a result of mis-segregation of chromosomes and can be induced by DNA and microtubule targeting drugs (24). Mitotic catastrophe is a trigger for cell death rather than a specific process by which cells die, and will usually lead to apoptosis in a p53-independent manner (198).

Apoptosis

Apoptosis is the most common and well-defined programmed cell death mechanism. It is a mechanism that allows other cells to take advantage of the nutrients in apoptotic cells via phagocytosis. Furthermore, it does not induce inflammation of the surrounding tissues, as is the case with necrosis. It can be induced by different types of stimuli. Apoptosis is a complex mechanism involving several pathways and proteins. An important class of proteins in the execution pathway are the cysteine proteases or the caspases (199). Upon the activation of this pathway the initiator caspases (8 or 9) are activated through oligomerization, leading to the subsequent cleavage of other down stream effector caspases. The activated caspases in turn cleave other substrates leading to characteristic biochemical and morphological changes such as: chromatin condensation, flipping of phosphatidylserine to the outer plasma membrane leaflet, cell membrane blebbing and formation of apoptotic bodies (200,201).

There are at least two signalling pathways leading to apoptosis, extrinsic and intrinsic. The extrinsic pathway involves the binding of ligands to cell surface receptors. Such external ligands include the FASL and tumour necrosis factor (TNF), which bind to their external surface receptors leading to a cascade of events. These begin with the formation of the death induced signalling complex (DISC), which in turn recruits caspase-8 and begins the cascade of procaspase activation (202). The intrinsic pathway is triggered by various

extracellular and intracellular stresses such as: growth-factor depletion, hypoxia, DNA damage and oncogene induction. Upon induction of the intrinsic pathway a series of biochemical events are induced that result in permeabilization of the outer membrane of mitochondria causing the release of cytochrome C and other pro-apoptotic molecules (203). This results in the formation of a large protein complex called the "apoptosome". The latter complex contains cytochrome C, caspase 9 and the apoptotic protease activating factor (APAF1), which, once activated, will promote a cascade of caspase activation (202). Of these steps, the permeabilization of the outer mitochondrial membrane is regulated by the anti-apoptotic members of the Bcl-2 family that can stop the cascade of events leading to apoptotic death (204). However, when cytochrome C is released, the cascade of caspase activation is irreversibly activated (205,206).

Finally, necrosis is an uncontrolled and non-regulated mechanism of cell death. This mode of cell death occurs when damage to a cell is too great and results in the loss of homeostasis and cell contents (207). Some of the morphological features resulting from severe cellular damage or necrosis include: membrane distortion, organelle degradation, and cellular swelling (208). Necrosis can be induced by physical or chemical damage. Physical damage includes anything that could affect the plasma membrane integrity, such as freezing, heating, and physical damage. Chemical hazards that lead to necrosis are more complex and not well understood but may include mechanisms such as membrane lipid oxidation, as seen with cells treated with BSO (123).

Generally, it is believed that chemotherapeutic drugs promote apoptosis in tumours. While *in vitro* this is often the case, *in vivo* other death pathways may be induced (209). Moreover, some chemotherapeutic drugs lead to senescence of the cells where they remain metabolically active but non-dividing (15,210). While, the goal of chemotherapy is to reduce tumour progression by killing tumour cells, growth arrest is also helpful since it leads to a reduction of tumour growth and stabilization of the disease.

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CHAPTER 2

Modulation of GSH levels in ABCC1 expressing tumor cells triggers apoptosis through oxidative stress

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ABSTRACT

The over-expression of ABCC1 transmembrane protein has been shown to cause multidrug resistance in tumor cell lines. ABCC1 is a member of the ABC transmembrane proteins that function as efflux pumps with diverse substrate specificity. Several endogenous cell metabolites, including the leukotriene C4 (LTC4) and glutathione (GSH) are substrates for ABCC1 protein. ABCC1 expression in certain tumor cells was demonstrated to confer hypersensitivity to glutathione modulating agents. In this report we have investigated the mechanism of collateral sensitivity seen in tumor cells over-expressing ABCC1 protein. The results of this study show that ABCC1 expression in tumor cells correlates with their hypersensitivity to various glutathione modulating agents, as demonstrated in H69AR-drug selected and HeLa/ABCC1-transfectant cells. This effect was triggered either through inhibition of GSH synthesis with BSO or by increasing ABCC1-mediated GSH transport with verapamil or apigenin. In addition, our results show that the hypersensitivity of ABCC1-expressing cells to BSO, verapamil or apigenin was preceded by an increase in reactive oxygen species (or ROS). A decrease in GSH level is also observed prior the increase in ROS. In addition, we show that hypersensitivity to the BSO, verapamil or apigenin leads to tumor cell death by apoptosis. Together, the results of this study demonstrate that ABCC1 potentiates oxidative stress in tumor cells through reductions in cellular GSH levels.

INTRODUCTION

Drug resistance is a major cause of treatment failure in clinical oncology [1]. Multidrug resistance (MDR) involves cross-resistance to a range of chemically unrelated agents with different cellular targets. The over-expression of ATP binding cassette (ABC) membrane proteins as causative proteins of MDR have been studied intensely over the past two decades [1]. These ABC proteins are, the P-glycoprotein 1 (P-gp1 or ABCB1), the breast cancer resistance protein (BCRP or ABCG2), and the multidrug resistance associated-protein 1 (MRP1 or ABCC1) [2]. Although the molecular mechanism by which the latter ABC proteins mediate the transport of structurally diverse drugs remains elusive, ABC proteins have been shown to bind and transport various ligands, including anti-cancer drugs, in an ATP-dependent manner.

ABCC1 was first isolated from a doxorubicin-resistant small lung cancer line H69AR [3], [4], [5] and [6]. The protein consists of 1531 amino acids, and when fully glycosylated, has a molecular mass of 190 kDa. ABCC1 is ubiquitously expressed in most normal tissues, with higher levels in lung, kidney, testis and blood mononuclear cells [6] and [7]. ABCC1 causes resistance to a broad spectrum of compounds including natural product drugs such as epipodophyllotoxins, vinca alkaloids, and certain anthracyclines [8], [9] and [10]. Furthermore, it transports a variety of substrates that are conjugated to glucoronide, sulfate, or glutathione [11], [12], [13] and [14]. Leukotriene C4 (LTC4), a conjugate of glutathione is the highest affinity substrate of ABCC1 [11] and [12]. Moreover, ABCC1 transports both reduced (GSH) and oxidized (GSSG) glutathione. GSH transport was observed in ABCC1-expressing H69AR cells, consistent with an earlier report by Cole et al. [15], which have shown that ABCC1-expressing H69AR cells have reduced cellular GSH levels compared to parental H69 cells. Later studies showed that GSH affinity for ABCC1 increases (from Km 10–20 mM to 10–100 μM) in the presence of certain drugs such as daunorubicin and vincristine [16] and [17]. This increase in GSH transport in the presence of these drugs appears to be mediated by co-transport mechanism [18]. The importance of GSH in ABCC1 activity is

illustrated by the reduction in transport of many substrates when GSH production is inhibited with l-buthionine (S,R)-sulfoximine (BSO) [18], [19] and [20]. BSO is the most specific and least toxic inhibitor of γ-glutamylcysteine synthetase (γ-GCS), thereby preventing de novo synthesis of GSH [21]. Several other compounds not transported by ABCC1 have been shown to stimulate ABCC1-mediated GSH export. Two such molecules that stimulate GSH transport are the calcium channel blocker verapamil (VRP) and the flavonoid apigenin (API) [22] and [23].

All aerobic organisms are subject to physiological oxidant stress as a consequence of aerobic metabolism. The production of ATP through oxidative phosphorylation leads to the formation of superoxide radicals (O_2^-). This radical can then form other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the hydroxyl radicals ($OH^{\cdot}$) [24]. These reactive compounds cause lipid peroxidation and can disrupt metabolic processes. GSH is the predominant defense against these toxic products of oxygen, particularly in mitochondria [25]. GSH is a major non-protein intracellular thiol that participates in redox reactions maintaining a reducing environment in the cell. Consequently, low GSH levels are sometimes associated with mitochondrial dysfunction and induction of apoptosis [26] and [27].

The present study explores some of the cellular consequences of GSH modulation in ABCC1 expressing cells. Our findings show that ABCC1-expressing tumor cell lines (H69AR or HelaABCC1 transfectant) are hypersensitive to a reduction in intracellular GSH. ABCC1-mediated drop in GSH levels appears to trigger apoptosis and is preceded by a burst of reactive oxygen species. This effect was triggered either by reducing GSH synthesis with BSO or by increasing ABCC1-mediated GSH transport with verapamil or apigenin. These observations demonstrate the mechanism by which ABCC1 expressing cells are hypersensitive to reductions in cellular GSH levels that can lead to avenues of targeting ABCC1 positive tumor cells.

MATERIALS AND METHODS

Materials. Protein-A Sepharose CL-4B and carrier-free Na125Iodine (100 mCi/mL) were purchased from Amersham Biosciences (Baie d'Urfe, Quebec). The ABCC1 polyclonal Ab was produced as describe below; polyclonal anti-actin [20], [21], [22], [23], [24], [25], [26], [27], [28], [29], [30], [31], [32] and [33] was purchased from Sigma (St. Louis, MO). NHS-ASA and ImmunoPure immobilized protein G were purchased from PIERCE (Rockford, IL). RPMI 1640 and α-MEM media were purchase from Gibco-BRL. Verapamil, apigenin, GSH, propidium iodide and MTT were purchase from Sigma–Aldrich. BSO was purchase form MP Biomedicals (Aurora, OH). H2DCFDA and FITC-conjugated Annexin V were purchase from molecular probes. Z-VAD-FMK was purchase from BD Pharmingen. C18 SepPak was purchase from Waters (Milford, MA). All other chemicals were of the highest possible quality.

Cell culture and transfection. The small cell lung cancer cell lines (H69, H69AR, and H69PR; from Dr. S. Cole at the Cancer Research Laboratories, Queen's University, Canada) were grown in RPMI 1640 media supplemented with 5% fetal bovine serum (or FBS). For the H69ARBcl-2 transfected cell line, G418 was added regularly at a concentration of 0.l mg/mL. Human cervical carcinoma cells (HeLa) and the ABCC1-transfected variants (HeLaABCC1; from Dr. P. Gros at Department of Biochemistry, McGill University, Canada) were cultured in α-MEM media supplemented with 10% FBS; while HeLaABCC1 was supplemented with 600 μg/mL G418 (Gibco-BRL) in addition to α-MEM media. H69/ARBcl-2 transfectant cells were generated by transfecting full-length human Bcl-2 cDNA. Briefly, H69/AR cells were plated (0.5 × 106) into 60 mm plates and transfected with a mammalian expression vector (Rc/RSV; Invitrogen, San Diego, CA) alone or containing full-length human Bcl-2 cDNA according to manufacturer's instructions using LipofectAMINE (Gibco-BRL). Twenty-four hours later, plates were treated with 0.1 mg/mL G-418 and the expression of Bcl-2 protein was monitored by Western blotting.

ABCC1 anti-sera and immuo-detection. A polypeptide encoding 15 amino acids from the C-terminal of ABCC1 (N-QRGLFYSMAKDAGLV-C) was synthesized using F-moc chemistry as previously described [28]. New Zealand Rabbits were immunized subcutaneously (sc) with a 1:1 ratio (v/v) of immunogen (thyroglobulin–peptide conjugate) and Freunds incomplete adjuvant (Sigma–Aldrich Inc.). Subsequent booster injections were administered at 2 weeks intervals and blood samples were drawn from each rabbit as previously described [29]. Antibody titer was checked by ELISA and Western blot analyses. For immuno-detection of ABCC1 and actin, 10 μg of total cell lysate was resolved on 10% acrylamide gels using the Laemmli gel system [30]. Proteins separated by SDS-PAGE were then transferred to nitrocellulose membrane [31] and probed with anti-ABCC1 (at 1:5000, v/v dilution in PBS), or anti-actin antibody (at 1:100, v/v dilution in PBS). Nitrocellulose membranes were washed with PBS and incubated with a 1:3000 (v/v) dilution of either goat anti-rat or goat anti-rabbit antibodies conjugated to horseradish peroxidase. SuperSignal West Pico chemoluminescent substrate (Pierce, Rickport, IL) was used to detect and develop the signal in Western blotting. For immuno-detection of ABCC1 protein in cell lysate, ELISA was performed on total cell lysate with 1 μg/well in 96-well plates. Wells were blocked for 2 h with 3% BSA in PBS at room temperature, followed by the addition of the first antibody (ABCC1 anti-peptide anti-serum at 1:500, v/v dilutions) for 16 h at 4 °C. Plates were washed with PBS and incubated with a HRP conjugated second antibody (goat anti-rabbit at 1:3000, v/v dilution) in blocking buffer minus sodium azide. The signal was developed with the addition of TMB substrate solution (Sigma–Aldrich), and then quantified by measuring the absorbance at 450 nm.

Cell survival assays. The effect of drugs on tumor cell growth was determined using an MTT colorimetric assay [32]. Cells were seeded in 96-well plates (at 2.5 × 103 cells/well for HeLa or HeLaABCC1 and 1 × 104 cells/well for H69,

H69AR, H69ARBcl-2, and H69PR cells) prior to the addition of various drugs. Cells were allowed to grow for 44 or 72 h in the absence or presence of increasing concentrations of BSO, VRP or API. The MTT dye was added to a final concentration of 50 μg/mL and cells were then incubated for another 4 h at 37 °C. The media containing residual MTT dye was carefully aspirated from each of the wells and a 200 μL aliquot of DMSO was added to each well to solubilize the reduced formazan dye. The effect of drugs on the growth of cells was determined from differences in absorbance between drug-treated cells versus untreated or solvent control. For cell rescue experiments, H69AR and HeLaABCC1 cells were incubated with increasing molar concentrations of API, VRP or BSO in media supplemented with various concentration of GSH, N-acetylcysteine (NAC) or Z-VAD-FMK, a generic Caspase inhibitor (BD Pharmingen).

Measurements of apoptosis by flow cytometry. Cell death from apoptosis was determine by quantifying the increase in Annexin V binding to surface exposed phosphotidylserine using Fluorescein isothiocyanate (or FITC) conjugated Annexin V. Briefly, cells were seeded in six-well plates (5 × 104 cells/well) and allowed to grow for 24 h in plates prior to the addition of API, BSO, or VRP. Cells were exposed to each drug for 1, 3, 9 or 24 h prior to cells harvesting and washing with buffer A (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl2, pH 7.4). Washed cells were then re-suspended in 100 μL of Annexin-binding buffer containing 5 μL FITC-conjugated Annexin V and allowed to incubate for 15 min. To gate-out dead cells due to necrosis, propidium iodide dye (PI, 1 μg/mL) was added to the cells prior to the FACS analysis, using an argon-ion laser source with detection filters set at 617 or 575 nm for PI or FITC-conjugated Annexin V detection, respectively.

Quantification of intracellular ROS. Cellular ROS were measured using the fluorogenic reagent ′2′ -dichlorodihydrofluorescein diacetate (H2DCFDA). Briefly, cells seeded in six-well plates (5 × 104 cells/well) were incubated with

API, BSO, or VRP for 1, 3, 9 or 24 h. ROS levels were determined by loading drug-treated cells with 10 μM H2DCFDA 30 min prior to cell harvesting. Cells were harvested, washed twice with PBS and then analyzed by FACS with fluorescence detection at 530 nm (BD FACS Aria™). Estimates of ROS following drug treatment were determined by measuring the change in mean fluorescence intensity using only live cells. Cells were gated using forward and side light scatter (FSC versus SSC) as well as propidium iodide exclusion.

Measurement of GSH levels. To determine the effect of drugs on GSH levels, a glutathione (GSH) detection kit from Chemicon international® was used. The experiment was performed according to manufacturer instructions with some modifications. Briefly, cells were incubated with each drug for 1 or 3 h, then washed and lysed. The cell lysates were centrifuged and supernatants were carefully removed and incubated with monochlorobimane (mcb) for 10 min. GSH levels were determined by measuring the fluorescence at 380/460 nm (ex/em).

Plasma membrane preparation and photoaffinity labelling. HeLa and HeLaABCC1 cells were cultured as described earlier. Cells were detached with trypsin-EDTA and washed with phosphate-buffered saline, pH 7.4 (PBS). The cell pellet was then resuspended in hypotonic buffer (1 mM $MgCl_2$, 10 mM KCl, and 10 mM Tris–HCl, pH 7.4) containing protease inhibitors (2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 μg/mL pepstatin A, 1 mM PMSF). Cells were lysed in a Dounce homogenizer and centrifuged at 10,000 × g for 5 min followed by a second centrifugation at 100,000 × g for 1 h. The final plasma membrane pellet was resuspended in 5 mM Tris–HCl, pH 7.4 containing 250 mM sucrose. Protein concentrations were quantified by Lowry et al. [33] and membranes were stored at −70 °C if not immediately used. Photoaffinity labeling of ABCC1 in plasma membranes with 125I labeled photoreactive analogue of GSH, IAAGSH, was performed as previously described [34]. Briefly, 1 μL of IAAGSH was added to 25 μg aliquots of plasma membrane in

a final volume of 20 μL of 5 mM Tris–HCl, pH 7.4, with 250 mM sucrose. Samples were incubated in the dark at room temperature for 30 min, followed by 10 min on ice and then UV irradiated at 254 nm for 10 min [35]. Photoaffinity labeled proteins were immunoprecipitated with anti-ABCC1 anti-serum or pre-immune serum and photoaffinity labeled proteins were immunoprecipitated with Protein-A conjugated Sepharose beads as previously described [36]. Proteins were eluted from Protein A beads in SDS-containing buffer and resolved by SDS-PAGE using Fairbanks gel system [37]. Gels with radiolabeled proteins were fixed, dried and exposed to Kodak BIOMAX MS film at −80 °C.

RESULTS

The initial characterization of the drug resistant H69AR cell line demonstrated that these cells displayed collateral sensitivity to BSO relative to their parental H69 cells. In light of more recent evidence for the role of ABCC1 in GSH transport, these findings implied that the over-expression of ABCC1 may reduce endogenous GSH levels rendering the cells hypersensitive or collaterally sensitive to BSO. The results in Fig. 1A shows that BSO has no effect on the H69 parental cells up to a concentration of 50 μM. Conversely, the H69AR cell line is extremely sensitive to BSO with IC50 of 0.6 μM. In order to confirm the role of ABCC1 in collateral sensitivity to BSO, the H69PR cell line was employed. H69PR are partial revertant from H69AR cells, selected without drugs for more than 36 months with very low ABCC1 expression [38]. When exposed to BSO, the H69PR cells were ten times less sensitive than H69AR, with an IC50 of 6.0 μM (Fig. 1A).

Although BSO is a highly specific inhibitor of γ-glutamylcysteine synthetase, it was of interest to determine if other modulators of cellular GSH levels also affect the survival of ABCC1-expressing tumor cells. Two such drugs, verapamil and apigenin, are known to decrease intracellular GSH through ABCC1-mediated efflux. Consistent with this premise, previous studies have demonstrated that ABCC1 transfected cells are more sensitive to verapamil, possibly due to increased GSH efflux [39]. To determine the effect of ABCC1-mediated GSH efflux on the growth of tumor cells, it was of interest to investigate the effects of both verapamil and apigenin on the above three cell lines. The results in Fig. 1B and C shows the effects of increasing concentrations of verapamil or apigenin on the growth of ABCC1-expressing drug resistant tumor cells (H69/AR) or ABCC1-negative parental (H69) and revertant cells (H69/PR). The IC50 values obtained with verapamil for H69, H69AR and H69PR cells were 100, 2.5 and 105 μM, respectively. Similarly, these cell lines displayed collateral sensitivity to apigenin with IC50 values of 23, 2.2 and 18 μM for H69, H69AR and H69PR, respectively. The differences

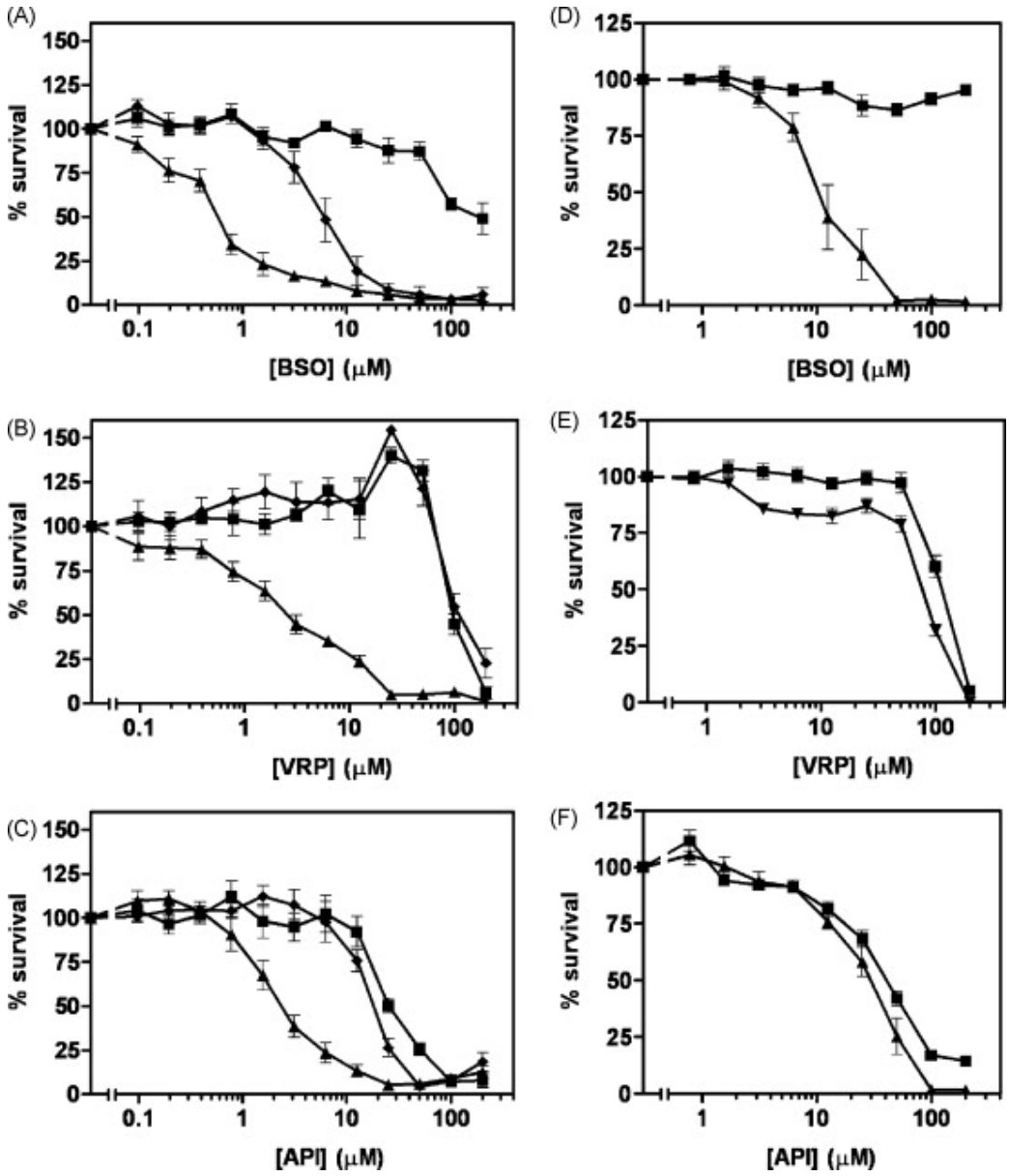

Fig. 1. Effects of BSO, VRP or API on the survival of tumor cells with or without ABCC1 expression. Cell growth with or without drugs was assessed by MTT assay. Parental or drug sensitive cells (H69 or HeLa cells; squares), ABCC1-expressing tumor cells (H69AR or HeLaABCC1 cells; triangles), and ABCC1-null H69PR cells (diamonds) were cultured in the presence of increasing concentrations of BSO (panels A and D), VRP (panels B and E) or API (panels C and F) for 48 h. Error bar represent standard deviation from three different experiments done in triplicate.

in collateral sensitivity of H69PR and H69 in the presence of BSO is noteworthy and argues for other cellular changes in these selected cells, in addition to increases in ABCC1 expression. However, given that other cellular changes than ABCC1 alone may be responsible for the observed effects of BSO, verapamil or apigenin, it was important to evaluate the effects of the latter compounds on ABCC1-transfected HeLa cells. Cell growth assays were performed on HeLa cells transfected with human ABCC1 (HeLaABCC1) to determine their sensitivity to BSO, verapamil and apigenin (Fig. 1D–F). The results in Fig. 1 shows HeLaABCC1 cells more sensitive to all three compounds, as compared to non-transfected HeLa cells. Surprisingly, however, BSO was dramatically more toxic to ABCC1-transfectants than verapamil or apigenin. The IC50 for BSO, verapamil, and apigenin in HeLaABCC1 cells were 9, 100 and 30 μM, respectively. By contrast, the IC50 of HeLa cells to the same drugs were 200, 125 and 50 μM, respectively. These results demonstrate that collateral sensitivity in these tumor cell lines can be mediated solely by ABCC1 expression. A two-way ANOVA test was used to determine the significance of the differences between HeLa and HeLaABCC1 cells exposed to verapamil and apigenin. These analyses found that overall the survival of each cell line was significantly different from one another. The effects of GSH modulating compounds shown in Fig. 1A–F are summarized in Table 1, where by the IC50 values for each GSH modulating compound in the different parental and ABCC1 expressing tumor cells are shown. Together these results indicate that ABCC1 plays an important role in collateral sensitivity to GSH modulating drugs. However, the effect of BSO on ABCC1-positive cells (HeLaABCC1 and H69/AR) appears similar, while that of verapamil or apigenin was significantly different between the two ABCC1-positive cell lines (see Fig. 1). Such differences are likely due to differences in ABCC1 expression between the two cell lines. The results in Fig. 2 shows ABCC1 expression in H69, H69AR, H69PR, HeLa and HeLaABCC1 as determined by Western blot (Fig. 2A) and ELISA (Fig. 2B). Fig. 2 shows the relative levels of ABCC1 expression and demonstrates that ABCC1 expression is highest in

Table 1.

Table 1 – Effects of GSH modulating drugs on tumor cells growth

Tumor cells	IC_{50} in μM		
	BSO	VRP	Api
H69	178	108	25
H69AR	0.6	2.4	2.3
H69PR	6.1	98.4	17.7
HeLa	–	114	40.1
$HeLa^{MRP1}$	10.1	74.4	29.0

Effects of GSH modulating drugs on tumor cells growth

H69AR cells, followed by lower levels in HeLaABCC1 cells. ABCC1 was not detectable in H69, H69PR, and HeLa cells (Fig. 2A). Collectively, the latter results demonstrate a correlation between the degree of collateral sensitivity and the level of ABCC1 expression, with H69AR cells expressing the highest levels of ABCC1 and the greatest sensitivity to verapamil, and apigenin.

Depletion of cellular GSH pools, through ABCC1-mediated GSH efflux (e.g. verapamil or apigenin) or inhibition of GSH de novo synthesis is thought to increase oxidative stress leading to cell death. To confirm this possibility, it was of interest to directly measure the levels of ROS and GSH in these cells and determine if they correlate with progression of cells to apoptosis. The results in Fig. 3A and D shows the effects of BSO, verapamil, and apigenin on H69AR and HeLaABCC1 cells as determined by Annexin V staining of tumor cells following a time course drug treatment. The results in Fig. 3A shows that apoptosis appeared quickly in H69AR treated with apigenin (1 h) and later with BSO and verapamil in HeLaABCC1 (9 h). As GSH function is to maintain a reductive state in mammalian cells, cellular levels of ROS were measured under the same conditions used to evaluate Annexin V staining or apoptosis. The amount of ROS was determined with the fluorescent reagent, H2DCFDA, as mean fluorescence of intact cells. The results in Fig. 3B and E shows a time-dependent increase in cellular ROS in H69AR and HeLaABCC1 after exposure to BSO, verapamil and apigenin, respectively. It is note worthy that the increase

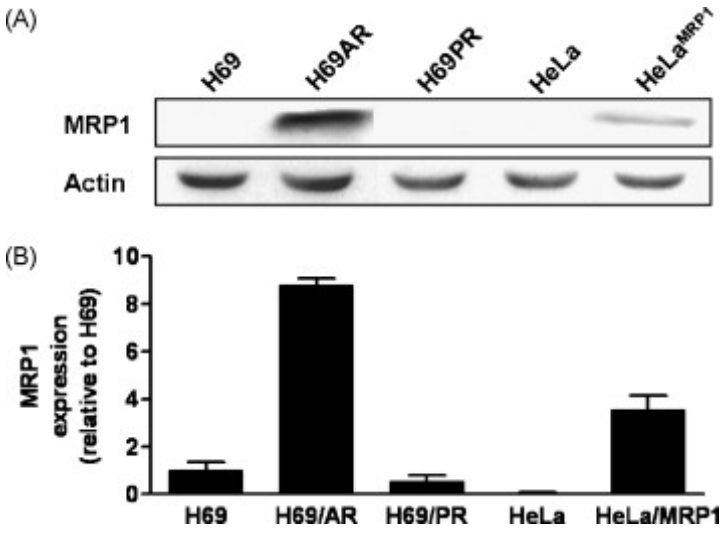

Fig. 2. ABCC1 protein expression in tumor cell lines. Total cell extracts from H69, H69AR, H69PR, HeLa, and HeLaABCC1 were resolved on SDS-PAGE and the expression of ABCC1 and actin were determined by Western blotting (panel A) or ELISA (panel B) using ABCC1 or actin-specific antibodies (see Section 2).

in ROS always appears before an increase in Annexin V staining, suggesting that accumulation of ROS may proceed the cellular changes leading to the induction of apoptosis. To confirm the effect of drugs on cellular GSH pools and the accumulation of ROS with GSH reduction, intracellular GSH levels were determined using the GSH reactive fluorescent dye, monochlorobimane. The results in Fig. 3C and F shows that all three drugs caused a significant decrease in cellular GSH levels. Again, this decrease in cellular GSH levels appeared before or simultaneously with the accumulation of ROS. Therefore, the reduction of GSH may be directly responsible for the accumulation of ROS and the eventual induction of apoptosis.

Given the correlation between the effects of drugs (BSO, verapamil and apigenin) on GSH depletion, rise in ROS and induction of apoptosis, it was important to determine if an increase in GSH pool in these cells could reverse

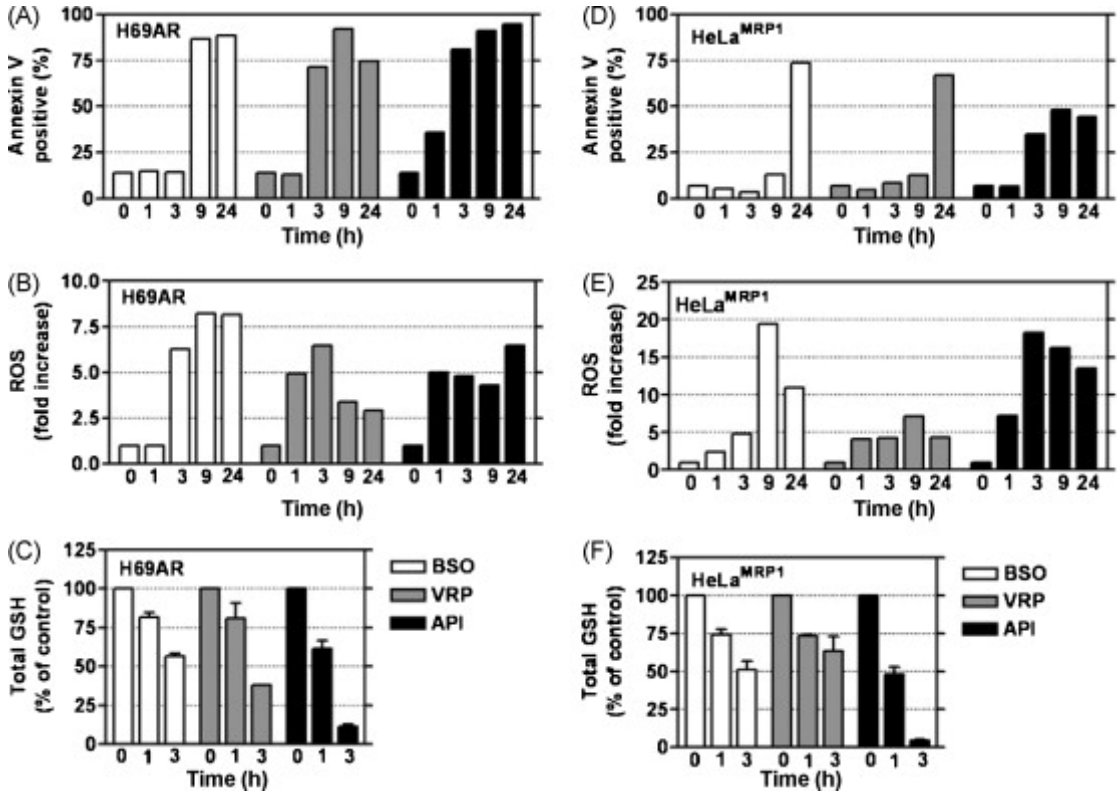

Fig. 3. Estimates of relative levels of apoptosis, ROS and GSH in H69AR or HeLaABCC1 cells without or with drug treatment. H69AR or HeLaABCC1 cells were treated with BSO (50 μM), VRP (50 μM) or Api (25 μM) for 1–24 h. Annexin V staining of cells was determined by flow cytometry (panels A and D). Panels B and E show relative levels of ROS in cells as determined from mean fluorescence of live cells loaded with H2DCFDA dye. The results from a single representative flow cytometry experiment are presented in panels A, B, D and E. Panels C and F show cellular GSH levels in drug treated relative to non-treated cells. Error bars represent standard deviation from results obtained in triplicate. The results above show a representative experiment repeated three times.

BSO, verapamil and apigenin-induced collateral sensitivity. The results in Fig. 3 are summarized in Table 2, showing the time (h) whereby exposure of tumor cells to BSO, VRP, or Api, results in a significant drop in cellular GSH levels, a five-fold increase in ROS and apoptosis in >40% of tumor cells.

Table 2.

Table 2 – Relative effects of GSH modulating drugs, a time-course, on GSH cellular levels, reactive oxygen species and apoptosis in tumor cells expressing ABCC1, relative to control

	Tumor cell lines					
	H69AR			$HeLa^{MRP1}$		
	BSO	VRP	Api	BSO	VRP	Api
Time leading to >20% drop of GSH levels (h)	1	1	1	1	1	1
Time leading to five-fold increase in ROS (h)	3	3	3	9	9	1
Time leading to 40% increase in apoptosis	9	3	3	24	24	9

Relative effects of GSH modulating drugs, a time-course, on GSH cellular levels, reactive oxygen species and apoptosis in tumor cells expressing ABCC1, relative to control

Previous studies have shown that free GSH is able to enter epithelial cells by an unknown mechanism [40]. Fig. 4A and B shows that co-incubation of HeLaABCC1 and H69AR cells with drugs together with increasing concentrations of GSH was able to counteract the toxicity of these drugs. In H69AR cells, 25 mM GSH was able to completely rescue cells from 12.5 μM BSO and 25 μM verapamil. The survival of cells exposed to 25 μM apigenin was increased from 0% to 35%. The survival of HeLaABCC1 cells exposed to 125 μM verapamil was completely rescued by 3.13 mM GSH. At 6.25 mM, GSH only partially improved the survival of HeLaABCC1 cells exposed to 25 μM BSO and 125 μM apigenin. Similar rescue effects were observed with the membrane-permeable GSH precursor, NAC (Fig. 4C and D), with near complete rescue in H69AR and a partial rescue in HeLaABCC1 cells.

To investigate further the molecular mechanism(s) of BSO, verapamil and apigenin on MRP-1 expressing tumor cells, the effect of the general caspase inhibitor Z-VAD-FMK on collateral sensitivity of drug treated H69AR cells was determined. The results in Fig. 5A shows that 100 μM Z-VAD-FMK completely reversed or inhibited verapamil-induced apoptosis; whereas the survival of H69AR cells exposed to 12.5 μM BSO was increased from 15% to 45%, while survival in the presence of 25 μM apigenin was increased from 10% to 48%. The effect of Z-VAD-FMK was also investigated in the H69AR cell line using H69AR cells transfected with human Bcl-2 full-length cDNA,

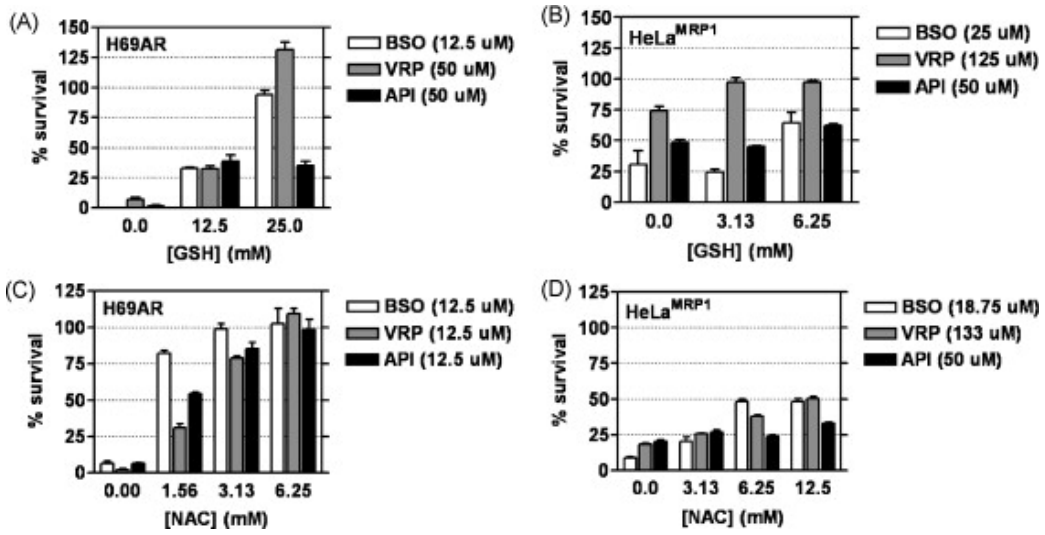

Fig. 4. Reversal of BSO, VRP or API effects on H69AR and HeLaABCC1 cells with GSH or NAC supplemented media. The effects of BSO, VRP and API alone or with exogenously supplemented GSH or NAC on the growth of tumor cells. Panel A shows the survival of H69AR cells without and with 12.5 or 25 mM GSH supplemented media. Similarly, Panel B shows the survival of HeLaABCC1 cells without and with 3.13 or 6.25 mM GSH supplemented media. Panel C shows the survival of H69AR cells without and with 1.56, 3.125 or 6.25 mM NAC supplemented media. Similarly, Panel D shows the survival of HeLaABCC1 cells without and with 3.13, 6.25 or 12.5 mM NAC supplemented media. Cell survival was determined with an MTT assay (see Section 2). Error bars represent standard deviation from results obtained in triplicate. The results above show a representative experiment repeated three times.

H69ARBcl-2. The results in Fig. 5B show that the increased expression of Bcl-2 in H69AR cells was protective from BSO, verapamil, and apigenin-induced apoptosis. Survival was increased by 20–40%, depending on the toxic drug. The greatest effect on survival (increase from 35% to 68%) was evident in cells exposed to BSO. These observations show that collateral sensitivity to drugs that reduce GSH levels in ABCC1 expressing cells may be in part due to an apoptotic pathway. It is important to point out that BCL2 transfection in

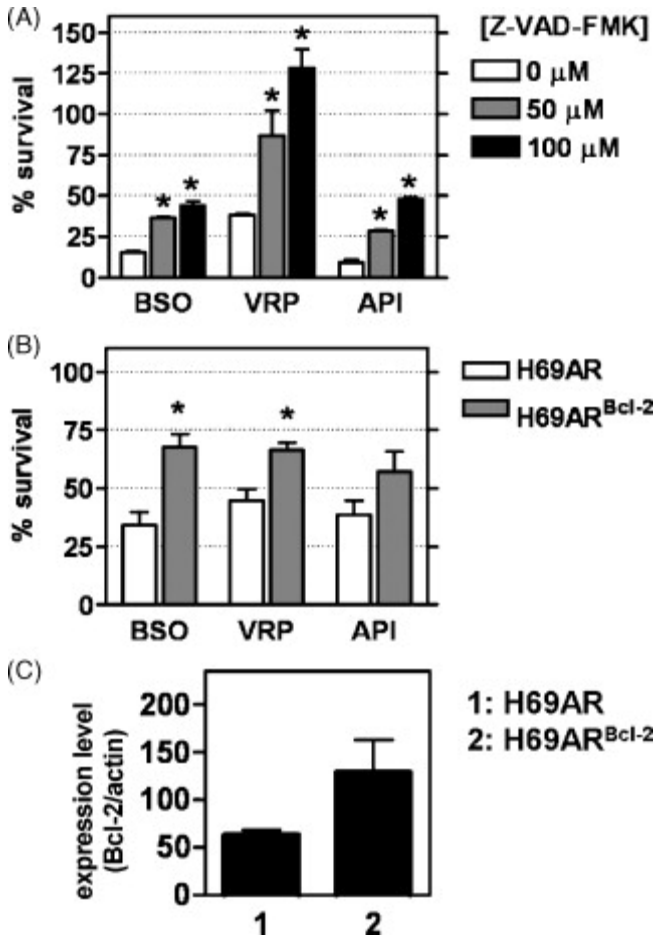

Fig. 5. Relative inhibition of BSO-, VRP- and API-induced apoptosis in H69AR and H69ARBcl2 cells. H69AR or H69ARBcl-2 cells were treated with BSO (12.5 or 6.25 μM), VRP (25 or 3.13 μM) and API (25 or 3.13 μM) in the absence or presence of the general caspase inhibitor, Z-VAD-FMK (at 0, 50 and 100 μM; panels A & B, respectively). Cell growth was determined by MTT after 24 h incubation. Panel C shows the relative increase in Bcl-2 expression in H69AR transfectants, as a ratio of actin expression between H69AR and H69ARBcl2 cells evaluated by densitometry of Western blot signals. Error bars represent standard deviation from results obtained in triplicate. Stared (*) bars show a significant increase ($P < 0.05$) relative to negative controls.

H69AR cells (H69ARBcl-2) did not alter the levels of ABCC1 relative to H69AR untransfected cells (results not shown), while Bcl2 expression was

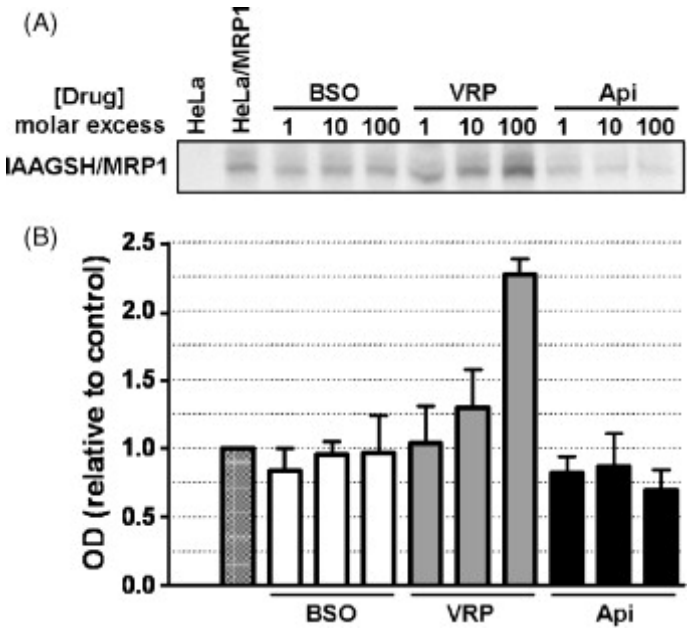

Fig. 6. Photoaffinity labeling of ABCC1 with IAAGSH. HeLa or HeLaABCC1 plasma membranes were photoaffinity labeled with 2 μM IAAGSH in the absence or presence of BSO, VRP and API. Drugs were added to plasma membranes containing IAAGSH at 1-, 10- or 100-molar excess. Panel B shows the relative change in photolabeling of ABCC1 with IAAGSH in the presence of the same drugs as evaluate by densitometry relative to solvent control. Error bars represent standard deviation.

increased by two-fold (Fig. 5C). Several studies [22] and [41] have shown that both verapamil and apigenin stimulate ABCC1-mediated efflux of GSH. We have previously demonstrated that verapamil-mediated increase of GSH efflux results in a significant increase in ABCC1 binding to GSH, as determined using a photoreactive analogue of GSH (or IAAGSH [34]). In light of our results in this study, together with our earlier observation with IAAGSH photoaffinity labelling of ABCC1, it was of interest to examine the effect of increasing concentrations of BSO, verapamil and apigenin on ABCC1 binding or photoaffinity labelling with IAAGSH. The results in Fig. 6A shows a significant increase (2.3-fold) in IAAGSH photolabeling of ABCC1 with 100-fold molar excess of verapamil. However, surprisingly, there was no increase in

the photoaffinity labelling of ABCC1 with IAAGSH in the presence of apigenin, as it has been previously shown [42] to increase ABCC1-dependent efflux of GSH. The absence of effect of BSO on ABCC1 photoaffinity labelling with IAAGSH is not surprising since BSO is not known to be a substrate of ABCC1 nor does it affect GSH efflux. Taken together, the results in Fig. 6 shows that verapamil and apigenin, although both enhance GSH efflux via ABCC1, do so by interacting with different sites on ABCC1.

DISCUSSION

ABCC1 has been shown to mediate the transport of endogenous cell metabolites and anti-cancer drugs via an ATP-dependent efflux mechanism [43]. One such endogenous substrate of ABCC1 is the tripeptide GSH, a major cellular detoxifying compound [44] and [45]. In this report we demonstrate that over-expression of ABCC1 contributes to cell death by oxidative stress through drug enhanced GSH efflux. Several studies have now demonstrated that GSH plays an important role in ABCC1 functions. In one instance, GSH is effluxed by ABCC1 pump, while in another GSH plays a catalytic role enhancing the transport of non-conjugated drugs. Consistent with its role as a substrate for, and modulator of ABCC1 efflux functions, we have recently demonstrated that GSH interacts with several domains in ABCC1, including drug transport domains [34]. Moreover, there is increasing evidence that ABCC1 expression in tumor cells or its lack-of (knockout studies) modulate cellular GSH levels. The three drugs tested in this study led to oxidative-stress induced apoptosis in ABCC1-expressing cells caused by inhibition of de novo synthesis of GSH (e.g. BSO), or increasing ABCC1-mediated transport of GSH (e.g. verapamil or apigenin). Specifically, our results show that ABCC1-expressing H69AR cells were hypersensitive to BSO (IC50 = 0.6 μM) relative to ABCC1-null parental H69 cells (IC50 = 200 μM). The revertant cell line, H69PR, was less affected with BSO (IC50 = 6 μM). The reduced toxicity of H69PR cells with BSO correlates with the lower expression level of ABCC1 in H69PR compared to H69AR. These findings indicate that the level of ABCC1 expression correlates with the degree of collateral toxicity to BSO; however other cellular changes in H69AR tumor cells unrelated to ABCC1 expression in these cells could not be ruled out. Hence, it was essential to examine the effects of BSO on cells where ABCC1 alone was differentially expressed. Concordantly, results with HeLaABCC1 cells display a far more pronounced sensitivity to BSO (IC50 = 10 μM) as compared to HeLa cells. Indeed, HeLa cells were unaffected by BSO up to a concentration of 200 μM. These findings demonstrate that BSO hypersensitivity is a consequence of ABCC1 expression in tumor cells. In this

respect, it would be of interest to examine the effects of BSO on normal tissues and organs that express high levels of ABCC1, such as in lung, kidney, liver, testis and blood mononuclear cells [6] and [7]. Phase I clinical trials using BSO alone or in combination with melphalan to reduce GSH intracellular pools have concluded that continuous infusion of BSO is relatively non-toxic at concentrations that cause 10–30% below normal baseline values of GSH [46] and [47]. Interestingly, the latter studies observed considerably lesser depletion of GSH in normal peripheral lymphocytes than that in tumor sections [46] and [47].

Earlier studies have shown that transport of GSH is enhanced by verapamil and apigenin [22] and [23]. Given this and the hypersensitivity to BSO, it was suspected that verapamil and apigenin could induce toxicity in ABCC1-expressing cells by reducing the intracellular concentration of GSH. Indeed, H69AR cells are 40-fold more sensitive to verapamil, and 10.5-fold more sensitive to apigenin, compared to H69 cells. Unlike BSO toxicity, the sensitivity of H69PR to these compounds was similar to H69. This highlights some of the difficulties in using MDR cell lines generated by drug selection. The drug selection process may alter the expression of other proteins. Consequently, the different levels of drug sensitivity in H69-derived cells may in-part be a product of phenotypic changes other than ABCC1 expression. To address this possibility, the effects of these compounds were tested in transfected HeLaABCC1 cells. As expected, HeLaABCC1 cells were more sensitive to verapamil and apigenin compared to HeLa cells. Together, these finding demonstrate clearly that collateral sensitivity to BSO, verapamil and apigenin is a direct consequence of ABCC1 expression.

As GSH plays an important role in cellular detoxification through its reducing potential [44] and [45], the accumulation of reactive oxygen species (ROS) may be the critical factor that initiates cell death due to BSO, verapamil and apigenin collateral sensitivity. High levels of intracellular ROS leading to intrinsic oxidative stress have been shown to cause cell death through apoptosis in different tumor model systems [48], [49], [50] and [51]. Our findings in this

study show that ROS and apoptosis are at least in part involved in cell death due to reductions in cellular GSH. Treatment of ABCC1-expressing cells with Z-FAD-FMK, a general caspase inhibitor, was able to significantly inhibit cell death. Furthermore, transfection of H69AR cells with the anti-apoptotic human bcl-2 gene was also able to improve survival and confirm that GSH-modulating agents are causative of tumor cell death through enhanced apoptosis. Z-FAD-FMK was most effective at inhibiting toxicity caused by verapamil; indicating that verapamil-initiated apoptosis is different from that of BSO or apigenin.

The reduction of cellular GSH levels, either through inhibition of synthesis or enhanced efflux, is likely to result in increased accumulation of ROS. Consistent with this latter link, our results in this study show that treatment of tumor cells with BSO, verapamil or apigenin causes a significant dose- and time-dependent increase in ROS. One of the most notable examples was the observed 19-fold increase in ROS in HeLaABCC1 cells following 24 h of treatment with BSO. Moreover, the increase in ROS production occurs prior to the appearance of apoptosis with all three compounds, in an ABCC1-dependent manner. Furthermore, reductions in GSH levels in these tumor model systems preceed apoptosis in cells exposed to BSO, verapamil or apigenin. The most striking reduction occurred in both H69AR and HeLaABCC1 cells treated with apigenin after 3 h, where GSH levels were reduced by approximately 90%. Remarkably, this finding correlates with previous studies that show apigenin to be one of the most effective stimulators of GSH transport by ABCC1 [22] and [42]. In all cases, the reduction in GSH starts 1 h after drug addition, resulting in the subsequent increase in ROS and the eventual appearance of apoptosis. If a reduction in intracellular GSH is indeed the initiating step leading to ROS production and subsequent apoptosis, it was reasoned that increasing GSH levels should negate the effects of these compounds and reverse collateral sensitivity. In support of the latter notion, the results in Fig. 4 shows that cells treated with BSO, verapamil and apigenin can be rescued by exogenously added GSH or NAC to the cell growth media. Again, this effect was most pronounced in BSO-treated H69AR cells in that

they were completely rescued with the addition of 25 mM GSH or 6.25 mM NAC. As expected, lower molar concentrations of NAC than GSH lead to a similar rescue effects, likely due to greater membrane permeability of NAC, a precursor of GSH. Indeed, it is not entirely clear how externally added GSH crosses the cell membrane. One possibility is the presence of low affinity GSH importer. Such GSH import has been described in the renal system whereby the sodium dicarboxylate transporter (SDCT-2) and the organic anion transporter OAT1/3 were demonstrated to mediate GSH import from extracellular milieu [40]. Although it is not know if these latter transporters are expressed in H69AR or HeLa cells, such transporters can mediate the GSH influx. Alternatively, high extracellular concentrations of GSH in ABCC1-expressing cells could competitively inhibit ABCC1-mediated GSH efflux or potentiate ABCC1-mediated influx of GSH. Taken together, this further demonstrates that GSH depletion is the primary or a significant mediator of collateral sensitivity in ABCC1 expressing tumor cells.

Although it is not entirely clear how verapamil and apigenin enhance GSH transport via ABCC1, it is thought that both compounds increase the ABCC1 affinity to GSH. Consistent with the latter hypothesis, we have previously shown that verapamil increases the affinity of ABCC1 for GSH binding, as evident by the substantial increase in ABCC1 photolabeling with IAAGSH [34]. However, surprisingly the results in this study show that while verapamil stimulates IAAGSH binding to ABCC1, apigenin did not increase ABCC1 binding to GSH. Although unexpected, it is possible that apigenin enhances ABCC1 efflux of GSH via a different mechanism than that seen with verapamil. Efforts are underway to compare differences in verapamil and apigenin binding on ABCC1.

In conclusion, this study points to a general oxidative stress that occurs in ABCC1-expressing cells as a consequence of ABCC1-mediated efflux of GSH. This state of oxidative stress can be further potentiated through the inhibition of the de novo synthesis of GSH or through enhanced GSH efflux.

Together, these findings provide an enticing way of specifically targeting drug resistant tumor cells that express ABCC1.

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Connecting Statement 1

Chapter 2 included a study that focused on the molecular mechanism of collateral sensitivity of ABCC1 (MRP1) expressing cells. Specifically, we proposed a mechanism where these MDR cells elicit increase sensitivity to GSH depletion. It was shown that BSO, Api and Vrp were all able to induce this mediated mechanism of collateral sensitivity. Some cells expressing ABCB1 (P-gp) are also collateraly sensitive to a variety of compounds. Among them, Vrp is the best studied. However, a complete understanding of the mechanism has no yet been realized. Chapter 3 examines the mechanism of collateral sensitivity of hamster P-gp expressing cells $CH^{R}C5$. This study explores the link between the phenomenon and the expression of P-gp and the involvement of the electron transport chain (ETC) mediated ROS, leading to apoptosis.

CHAPTER 3

P-glycoprotein (ABCB1) Modulates Verapamil Collateral Sensitivity of Multidrug-resistant Tumor Cells

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(manuscript in preparation)

ABSTRACT

Elevated expression of P-glycoprotein (P-gp1, ABCB1) in tumor cells causes multidrug resistance via active efflux of drugs across the cell membrane. However, the overexpression of P-gp1 in some tumor cell lines has been associated with increased sensitivity, or "collateral sensitivity", of multidrug resistant cells to specific drugs, including the calcium channel blocker verapamil. It was recently demonstrated by this laboratory that collateral sensitivity to verapamil correlates with its stimulation of P-gp1 ATPase, and is reversed by inhibitors of P-gp1 ATPase (eg., PSC 833 and Ivermectin) (Biochemistry. 2003; 42:12163-73). In this report, it is demonstrated for the first time that P-gp1 expression in drug-resistant cells is directly linked to collateral sensitivity. P-gp1-specific siRNA significantly down-regulated P-gp1 expression in CH^RC5 cells within 24 h post-transfection of siRNA, leading to increased sensitivity of CH^RC5 cells to paclitaxel and doxorubicin but not to cis-platinum. Interestingly, down-regulation of P-gp1 expression greatly reversed collateral sensitivity to verapamil. We also demonstrate that CH^RC5 cells elicit a higher basal membrane ATP consumption. Moreover, we show that known inhibitors of electron transport chain (ETC), rotenone and antimycin A, also lead to hypersensitivty in addition to synergize with verapamil induced collateral sensitivity. Moreover, we show that known inhibitors of ETC, rotenone and antimycin A, synergize with verapamil induced collateral sensitivity through ROS induction. Taken together, the results of this study show for the first time a direct link between P-gp1 expression and collateral sensitivity of drug-resistant cells, through oxidative stress due to a rise in ROS.

INTRODUCTION

A major problem in cancer treatment is the outgrowth of multidrug-resistant (MDR) tumor cells following chemotherapy treatment of patients. Acquisition of multidrug resistance is also observed in tumor cell lines selected *in vitro* with anticancer drugs. *In vitro* the MDR phenotype is frequently coupled to overexpression of one or more ATP- Binding Cassette (ABC) transporters. Three such transporters [P-glycoprotein (P-gp1, or ABCB1), the Multidrug resistance protein (MRP1 or ABCC1) and the breast cancer resistant protein (BCRP or ABCG2)] have been shown to be causative of MDR in tumor cell lines (36). These membrane transporters are members of a large family consisting of 48 ABC transporters found in humans (211). In tumor cells, the overexpression of the above ABC transporters mediates the efflux of drugs in an ATP-dependent mechanism (212). In normal tissue, P-gp1 expression has been demonstrated in most cells with secretory or absorption functions (61), in intestinal epithelia at the apical membrane, in the kidney proximal renal tubule at the brush border membrane, in the liver at the canalicular membrane of hepatocytes, and in the brain at the luminal surface of capillary endothelial cells (213,214). Consequently, based on P-gp1 normal expression, it is thought to play a significant role in normal tissue absorption and secretion of xenobiotics or toxic metabolites (29). P-gp1 overexpression has been shown to correlate with reduced overall patient survival in some cancers (36). P-gp1 expression in untreated tumors varied significantly [e.g. 7% for brain tumors and sarcomas, but up to 100% for liver cancers; (215)] with a mean frequency of roughly 40% for all tumor samples. However, the frequency of P-gp1 expression was increased following treatment, while the percent increase in frequency varied between different malignancies, with the highest increase in lung and lowest in sarcomas (215). In an effort to overcome P-gp1 mediated drug resistance, much effort was placed on the development of competitive drugs to sensitize resistant cells (216). Beginning with verapamil, as the first MDR-reversing agent, other drugs [e.g., the non-immunosuppressant cyclosporin A analogue (PSC833 or valspodar), the multi-ABC pump inhibitors, VX-710 and GF120918] were later

identified as more potent MDR-reversing agents (217,218) (89,219,220). Clinical trials with all three MDR-reversing agents (e.g. PSC833, VX-710 and GF120918) have led to a significant increase in the area under the curve (AUC) of anticancer drugs at the expense of considerable haematological, neuropathic, and hepatic toxicities but without a significant gain in therapeutic index of anticancer treatment (221). Thus while P-gp1 expression has been demonstrated in untreated and treated tumors, and as such represents an excellent therapeutic target for the treatment of cancer, the use of competitive inhibitors of P-gp1 has been halted due to marked toxic effects associated with inhibition of its normal function.

Drug-resistant cells expressing high levels of P-gp1 may display hypersensitivity to specific drugs that are non-toxic to normal or P-gp1-negative tumor cells, a phenomenon referred as “collateral sensitivity” (2). Among these drugs are the calcium channel blocker Verapamil, non-ionic detergents and steroids (6) (222). However, not all P-gp1-expressing drug-resistant cells display collateral sensitivity to the above agents. We have previously demonstrated a correlation between verapamil hypersensitivity of drug-resistant cells and P-gp1 ATPase activity (7); however, direct correlation between P-gp1 expression and collateral sensitivity has been lacking. The need for this direct correlation was reinforced by the fact that not all drug-resistant P-gp1-positive cell lines show collateral sensitivity to verapamil. More recently, a report by Ludwig *et. al.* (94) described collateral sensitivity in P-gp1-expressing cells independent of P-gp1 ATPase stimulation. In this study, we demonstrated a direct link between P-gp1 expression and verapamil-induced collateral sensitivity in drug-resistant cells. Using the RNA interference approach, we demonstrated verapamil-induced collateral sensitivity can be reversed when P-gp1 expression is down-regulated. We also demonstrate that the presence of P-gp1 at the plasma membrane induces an important basal ATP consumption leading to hypersensitivity to ETC inhibitors. The latter is consistent with our finding in this study, whereby ETC inhibitors (known to induce ROS) synergized with verapamil. Taken together the findings in this

study implicate P-gp1 directly and provide a molecular approach to investigate a more detailed molecular understanding of this phenotype in MDR-resistant cells.

MATERIALS AND METHODS

CH^RC5 Clones preparation. Dilution was prepared in order to obtained 50 cells that were distributed in a 96 wells plate (200 µl media containing 5 µg/ml of colchicines per well). Clones were allowed to growth until colonies formed. The cells were verified under optical microscope to check for multiple colonies, such a case the well was discarded. Colonies were harvested then expended in 12 wells plate and then in T25 flask.

Cell Growth Assay. Cells transfected with siRNA sequences were seeded in triplicate into 96-well plates at $5x10^3$ cells/well 48 h post-transfection. For all cell growth assays including transfected cells or unless indicated otherwise, following 24 h incubation, cells were exposed to increasing concentrations of drugs and allowed to grow for 72 h. When used in combination PSC833, rotenone, antimycin A and H_2O_2 were added 4 hours prior the other drug. The assay was developed by the addition of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye to a final concentration of 0.5 mg/ml into each well and plates were incubated at 37°C for 4 h. The MTT crystals were dissolved with 200 µL of DMSO. Cell growth was determined from the absorbance values measured at 570 nm relative to control or untreated cells.

Cell Culture and siRNA Transfection. AuxB1 and drug-resistant selected cell line (CH^RC5, a kind gift from Dr. V. Ling, at the BC Cancer Centre, in BC, Canada) were grown in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO_2 without and with 5 µg/ml colchicine. For siRNA transfection, siRNA duplex sequences corresponding to the Chinese Hamster mdr1 sequence [5'AGAGAAGAAACCAGUGGUC3' (sense) and 5'GACCACUGGUUUCUUCUCU3' (antisense)], or negative control (Mock) siRNA duplex sequences [5'UAAAGAGUUACCCUAGUUUCA3' and 5'UGAAACUAGGGUAACUCUUUA3'] were synthesized by Invitrogen Life technology Inc. Cells in exponential phase, at 50% confluency, were transfected

with 1 nmole siRNA complexed with lipofectamine 2000 (Invitrogen, Burlington, On, Canada) in serum-free α-MEM according to manufacturer's protocol, with some modification. Briefly, prior to transfection, cells were washed once with serum-free α-MEM and incubated with the transfection mix for 6 h. Cells were washed with serum-containing media and incubated with or without drugs for the rest of the experiment.

Protein Extraction and Immunodetection. siRNA transfected cells ($1X10^6$) were harvested every day for 6 days post-transfection. Cells were washed three times with PBS and lysed with 50 µl of lysis buffer (50 mM Tris-Cl pH 7.8, 150 mM NaCl, 1% NP-40, containing 1% protease inhibitor cocktail). The cell lysate was centrifuged at 13,000 g for 20 min and the supernatant was carefully removed. For immunodetection by Western blots, 25 µg sample of cell lysate was resolved on Fairbanks gels (54) and resolved proteins transferred onto nitrocellulose membrane (223). The same nitrocellulose membrane was probed for P-gp1 and actin expression using a P-gp1-specific monoclonal antibody (265/F4, Abcam, Cambridge MA) and actin specific polyclonal antibody (20-33, Sigma, Oakville, On, Canada) at 1:250 (v/v) dilution, respectively. HRP-conjugated goat α-mouse or α-rabbit IgGs (BioRad, Hercules, CA) were used at 1:3000 v/v dilution as second antibodies. HRP detection was performed using Immobilon Western detection kit (Millipore, Billerica, MA) according to manufacturer instructions. For ELISA, 2 µg of cell lysate was used to coat the wells of 96-well plates in 10 mM ammonium bicarbonate buffer, pH 10, for 2 h at room temperature. The wells were washed three times with PBS and blocked with 1% BSA in PBS for 2 h. The assay was developed with the same primary (265/F4 mAb or α-actin pAb), and secondary (HRP-conjugated goat α-mouse or α-rabbit IgG) antibodies diluted in blocking buffer. P-gp1 expression was determined by absorbance at 450 nm relative to actin levels and AuxB1 cell lysate used as background for P-gp1.

Plasma Membrane Preparation and ATPase Assay. AuxB1 and CH^RC5 cells were washed with PBS and resuspended in 10 mL hypotonic buffer (10mM HEPES-Tris pH 7.4, 5 mM EDTA, 5 mM EGTA, and 2 mM dithiothreitol) containing protease inhibitors (2 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL pepstatin). Cells were ruptured with a glass homogeniser (Kontes, Vineland, NJ) and plasma membrane isolated by differential centrifugation, with a final centrifugation at 45000x*g* for 60 min (7). The latter membrane pellets were resuspended in 1 mL buffer using a 27-gauge syringe and stored at –80°C. P-gp1 ATPase activity was determined by quantifying the release of inorganic phosphate from ATP as previously described (7). P-gp1 specific ATPase activity was determine in the presence of mitochondrial, Ca^{2+} dependant and Na^+ / K^+ dependant ATPase inhibitor; 10 mM NaN_3, 4 mM EGTA and 2 mM Ouabain. Each condition represents experiment done three independent times with six replicates.

Statistics. All graph and stats were performed using Graphpad prism version 4. All statistics represent the Student's t test.

RESULTS

Verapamil Collateral Sensitivity in CH^RC5 Clones. Even though the selection of the CH^RC5 was clonal (52) and other groups (224,225) observed the same pattern of collateral sensitivity to verapamil, the unusual biphasic shape of the survival curve suggests the possibility of a mixed population. To eliminate this possibility, CH^RC5 cells were recloned through serial dilution. Ten clones were randomly isolated and treated with Verapamil. The results in figure 1 show that all clones display the same pattern of toxicity as the (non-recloned) CH^RC5 cells. Together, these results and earlier reports of biphasic response to verapamil negate the possibility that the hypersensitivity of CH^RC5 cells is due to a mixed cell population. As we observed previously, the collateral sensitivity to verapamil follows an atypic toxicity curve shape (7). The first peak of toxicity occurs at ~3 µM followed by a toxicity decrease. Verapamil concentrations become toxic again (this time for the parental cells as well) around 100 µM. This indicates the presence of two or more effects or mechanisms. The initial effect, specific for CH^RC5, represents the collateral sensitivity, while the second effect, non cell specific, remains undetermined but could be related to verapamil associated Ca^{2+} channel blocking.

P-gp Expression After MDR1 siRNA Treatment. In this report the direct role of P-gp1 in collateral sensitivity was investigated by down-regulating P-gp1 expression with RNAi. siRNA sequences used to down-regulate of P-gp1 expression were designed according to previously published sequences for human P-gp1 (226). Figure 2A shows the down-regulation of P-gp1 expression in CH^RC5 cells transfected with P-gp1-siRNA, as quantified by ELISA. A decrease in P-gp1 expression was observed as early as day 1 post mdr1-siRNA transfection compare to non-transfected CH^RC5 (figure 2A), with a maximum decrease in expression seen at day two posttransfection. P-gp1 expression begins to recover, reaching normal expression on day six post-transfection. No reduction in P-gp1 expression was observed in CH^RC5 cells

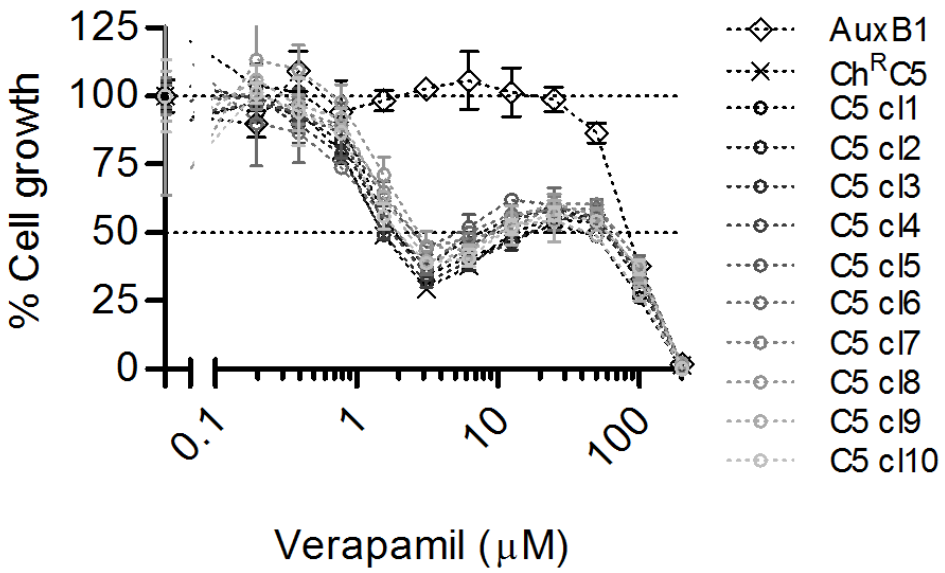

Figure 1. Verapamil collateral sensitivity in CH^RC5 **clones.** Cell growth was assessed by MTT assay. Parental or drug-sensitive cells (AuxB1 cells; +), P-gp-expressing tumor cells (CH^RC5 cells; X), and the CH^RC5 clones (C5 cl). Cells were grown in the presence of increasing concentrations of Verapamil for 72 h. Error bars represent standard deviation from a single experiments done in triplicate.

transfected with an irrelevant (or Mock) siRNA. The ELISA results were further confirmed by Western blotting using P-gp1- and actin-specific antibodies (figure 2B). To our knowledge this is the first demonstration of siRNA-mediated P-gp1 knockdown in the hamster model.

P-gp Knock Down MDR Reversal. To confirm that the decrease in P-gp1 expression is reflected phenotypically, we examined the sensitivity of untransfected, mock and P-gp1 siRNA transfected CH^RC5 cells to several anticancer drugs that are known substrates of P-gp1. Figure 3 shows the growth of untransfected, mock and P-gp1 siRNA transfected CH^RC5 cells in the

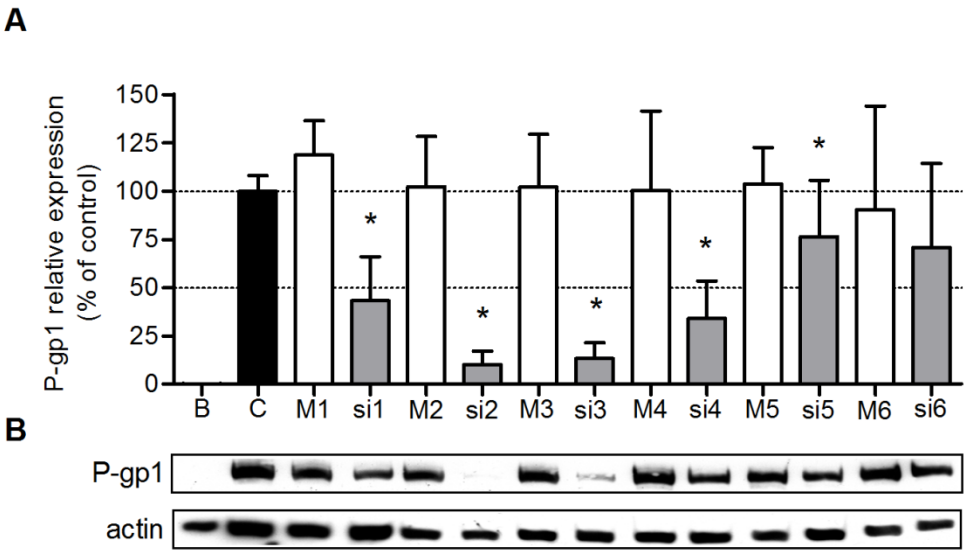

Figure 2. Down-regulation of P-gp1 expression with siRNA. Relative levels of P-gp1 expression was measured in AuxB1, CH^RC5, CH^RC5 transfected with Mock siRNA (M) and CH^RC5 transfected with P-gp1 siRNA (si). The numbers next to the above letter designations represent days post-siRNA transfection. Protein levels were evaluated by ELISA (panel A) or Western blotting (panel B) using P-gp1-specific monoclonal antibody. ELISA was evaluated in 96 well plates using TMB and quantity depicted relative to CH^RC5 normalized to actin concentration. Western blot was performed after protein resolution on Fairbanks system. Error bars represents Standard deviation of three experiments done in triplicate. *, $P < 0.05$, statistically significant difference compared with CH^RC5 P-gp1 protein level.

presence of increasing concentrations of paclitaxel, doxorubicin (P-gp1 substrates) or cis-platinum (not a P-gp1 substrate) (29). The results in figure 3 show a strong increase in sensitivity of P-gp1 siRNA CH^RC5 transfected to paclitaxel and doxorubicin versus mock siRNA transfected and untransfected CH^RC5 cells. The latter results show clearly, that down-regulation of Pgp1 in CH^RC5 cells with P-gp1 siRNA causes a significant decrease in IC_{50} to paclitaxel and doxorubicin relative to CH^RC5 untransfected or mock siRNA transfected cells (Figure 3). It is note worthy that in spite the down-regulation

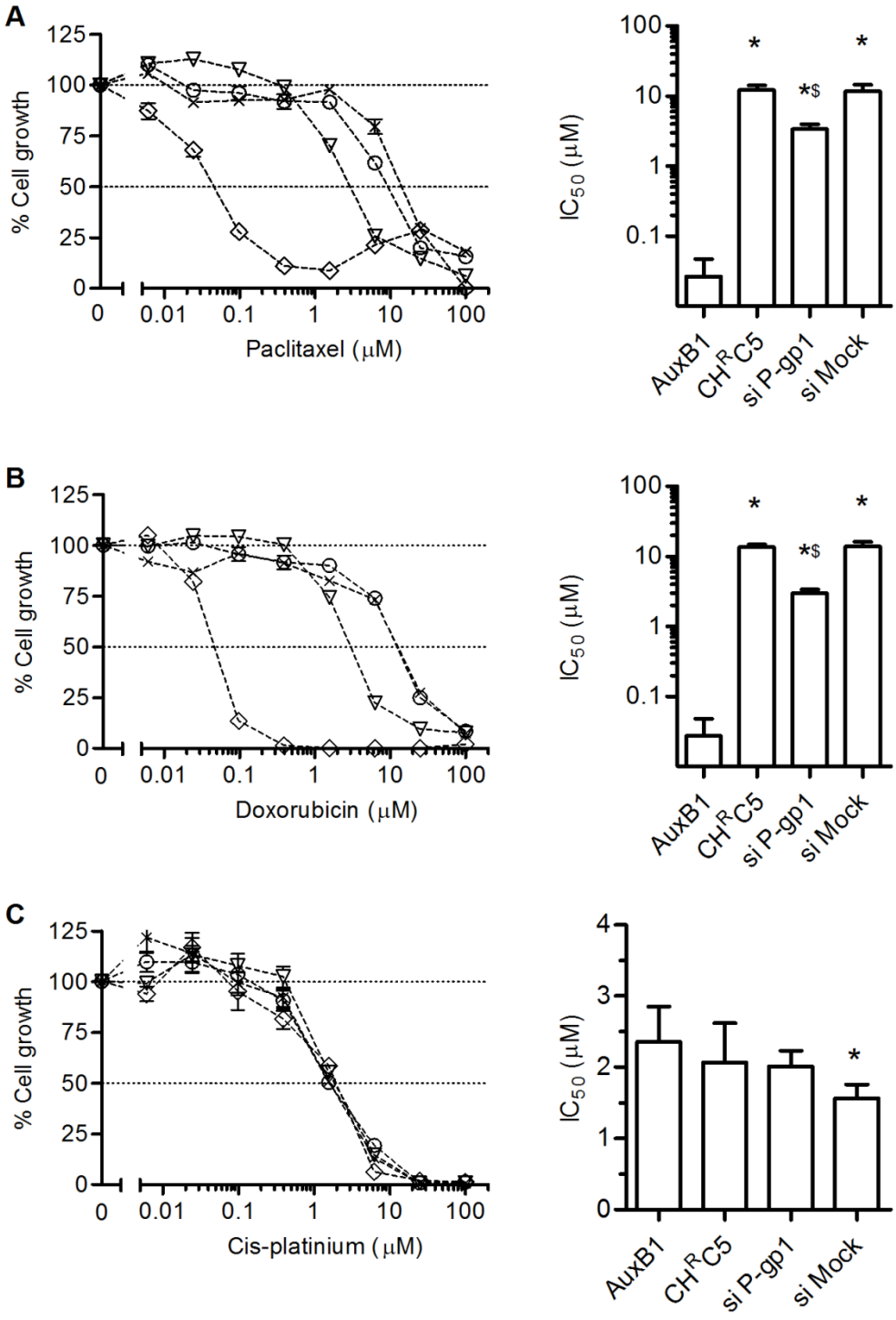

Figure 3. Effect of Paclitaxel, Doxorubicin or Cis-platinum on the survival of tumor cells with or without P-gp1 expression. Cell growth with or without drugs was assessed by MTT assay. Parental or drug-sensitive cells (AuxB1

cells; open diamond), P-gp expressing tumor cells (CH^RC5 cells; filled diamond), P-gp1-siRNA transfected CH^RC5 cells (inverted triangle) and Mock siRNA transfected CH^RC5 cells (triangle), were grown in the presence of increasing concentrations of Paclitaxel (panel A), Doxorubicin (panel B) or Cis-Platinum (panel C) for 72 h. Error bars represent standard deviation from at least two different experiments done in triplicate. * and ¥, $P < 0.01$, statistically significant difference compared to AuxB1 (*) or CH^RC5 (¥).

of P-gp1 in CH^RC5 siRNA transfected cells and the concomitant increase in sensitivity of these cells relative to mock transfected, the former cells remain less sensitive to paclitaxel or doxorubicin than AuxB1 (approximately 150- to 170-fold more resistant respectively). One possibility is that other mechanisms than P-gp1 in these cells relative to AuxB1 may mediate resistance to paclitaxel and doxorubicin. Alternatively, the observed residual resistance in P-gp1-siRNA CH^RC5 transfected cells may be due to the transient nature of P-gp1 siRNA-mediated down regulation (e.g., 24 to 48h) and the duration of the MTT assay (e.g., 72h). Indeed, the results in figure 1 show clearly that P-gp1 expression begins to increase on the 3rd day following P-gp1 siRNA transfection. However, as expected, down-regulation of P-gp1 in CH^RC5 cells transfected with P-gp1 siRNA did not show increased sensitivity to cis-platinum, a non-P-gp1 substrate drug. More importantly, treatment of CH^RC5 cells with P-gp1-siRNA or Mock siRNA did not cause a significant shift in IC_{50} of cis-platinum relative to the CH^RC5. A weak but significant decrease can be observed with the Mock transfected when compare to the parental AuxB1: IC_{50} ± SD of 2.4 ± 0.5 and 1.6 ± 0.2 respectively. Even though not expected, this effect does not appear to be important since it is not observed relative to the CH^RC5 in which the transfection was performed. Taken together, these results demonstrate that transient down-regulation of P-gp1 expression in CH^RC5 cells causes a large increase in the sensitivity of these cells to paclitaxel and doxorubicin (P-gp1-drug substrates) but not to cis-platinum (non-P-gp1 drug) therefore validating this approach in studying P-gp1-mediated effects.

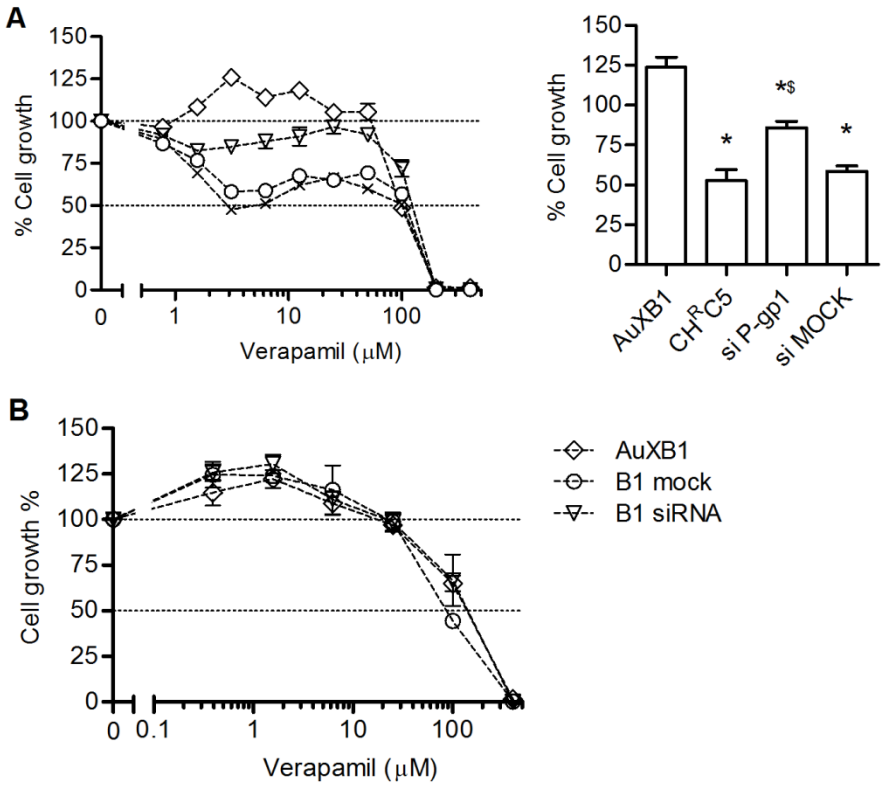

Figure 4. Effect of verapamil on the survival of tumor cells with or without P-gp1 expression. Cell growth was assessed by MTT assay. Parental or drug-sensitive cells (AuxB1 cells; open diamond), P-gp-expressing tumor cells (CH^RC5 cells; filled diamond), P-gp1-siRNA transfected CH^RC5 cells (inverted triangle) and Mock siRNA transfected CH^RC5 cells (triangle), were grown in the presence of increasing concentrations of Verapamil for 72 hrs (panel A). Parental (AuxB1 cells; square), P-gp1-siRNA transfected AuxB1 cells (inverted triangle) and Mock siRNA transfected AuxB1 cells (triangle),were grown in the presence of increasing concentrations of Verapamil for 72 h (panel B). Error bars represent standard deviation from two different experiments done in triplicate. * and ¥, $P < 0.01$, statistically significant difference compared to AuxB1 (*) or CH^RC5 (¥).

P-gp1 Knock Down Collateral Sensitivity Reversal. Having established a significant down-regulation of P-gp1 in CH^RC5 drug-resistant cells with P-gp1-specific siRNA, we examined how such down-regulation of P-gp1 affects the collateral sensitivity of these cells to verapamil. The results in figure 4 A show that down-regulation of P-gp1 in CH^RC5 cells decreased significantly the sensitivity of CH^RC5 cells to verapamil relative to mock transfectant or untransfectant CH^RC5 cells. The same treatment on the AuxB1 cells did not have any effect as seen in figure 4 B, confirming the specificity of the siRNA. These results show for the first time a direct correlation between P-gp1 expression and collateral sensitivity to verapamil. Of interest is the almost complete reversal of verapamil collateral sensitivity following down-regulation of P-gp1 expression alone passing from 52.7 ± 6.6 to 85.7 ± 4.1 % of cell growth.

P-gp ATPase Basal Activity and Stimulation by Verapamil. We have previously demonstrated a correlation between collateral sensitivity and the effect of verapamil on P-gp1 ATPase activity. Knowing that CH^RC5 expresses a high amount of P-gp1 (up to 12 % total plasma membrane protein) (227), we were interested to see if this abundance of a energyvorous protein at the membrane would be enough to change the basal membrane ATPase activity in these cells. The results of the experiment in figure 5 A show clearly that CH^RC5 membrane ATPase activity is three times that of AuxB1. As we and others already published, in figure 5 B we see a strong P-gp1 ATPase stimulation by verapamil on CH^RC5 purified plasma membranes. AuxB1 results are not shown since P-gp1 specific activity was at the background level and not influence by the presence of the compounds. As previously demonstrated (7,82,83), the drug caused a significant increase up to 2.5-fold in P-gp1 ATPase.

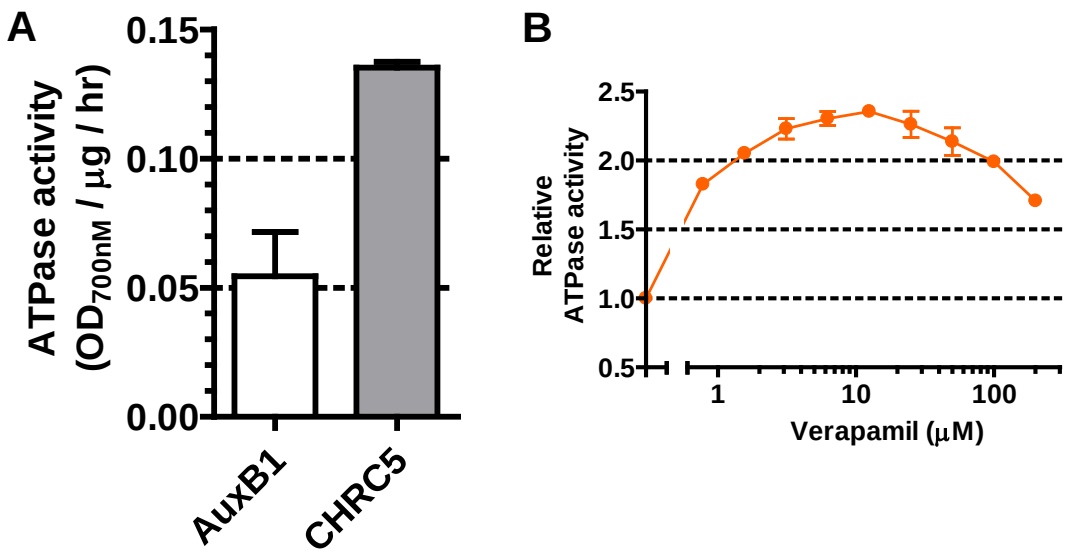

Figure 5. Total ATPase activity and effects of verapamil on P-gp1. ATPase activity was measured using purified AuxB1 and $CH^{R}C5$ plasma membranes (panel A). In panel B, P-gp1 specific ATPase was measured using purified $CH^{R}C5$ plasma membranes exposed to increasing concentration of verapamil. Error bars represent standard deviation from at least one representative experiment done in triplicate. *, $P < 0.01$, statistically significant difference compared to AuxB1.

P-gp Dependent Collateral Sensitivity to Electron Transport Chain Inhibitors and synergy with Verapamil.

In our previous studies we attributed the collateral sensitivity phenomenon to the increase of ATP consumption (7). As we saw in figure 5, $CH^{R}C5$ cells elicit a three time higher basal ATPase activity at the plasma membrane level compared to its parental cell. It was of interest to look if this higher energy demand would pre-sensitize these cells to main energy supply inhibition with ETC blockage. Therefore, we treated these cells with the known complex I and III inhibitors rotenone and antimycin A, respectively. As seen in figure 6 A and B, $CH^{R}C5$ cells are much more sensitive than the parental. To evaluate if this collateral sensitivity was due to the to P-gp expression, we conducted siRNA against MDR1 and submitted the cells to toxic concentration of rotenone (1

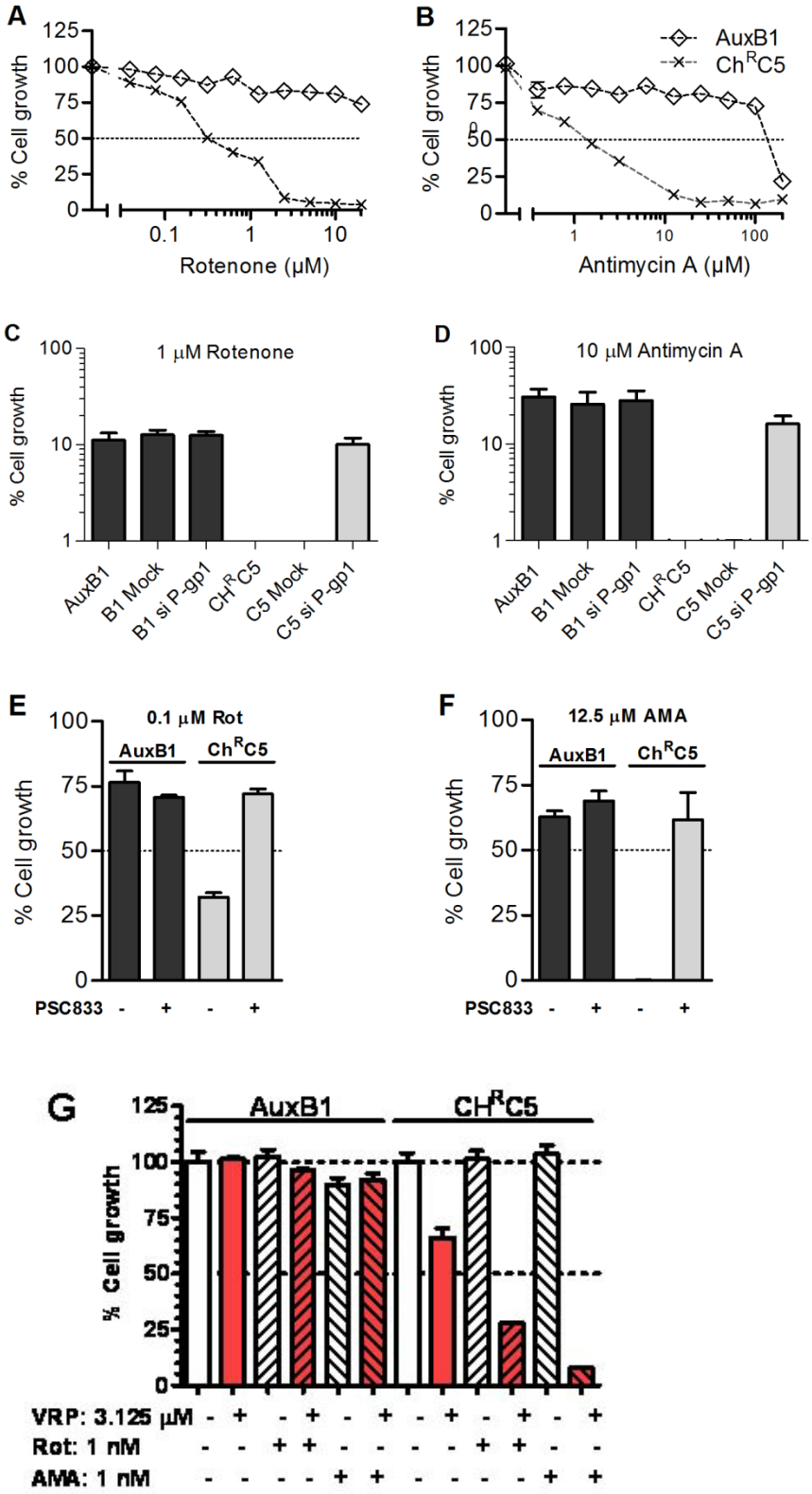
A
% Cell growth
Rotenone (µM)
B
AuxB1
ChRC5
% Cell growth
Antimycin A (µM)
C
1 µM Rotenone
% Cell growth
AuxB1
B1 Mock
B1 si P-gp1
ChRC5
C5 Mock
C5 si P-gp1
D
10 µM Antimycin A
% Cell growth
E
0.1 µM Rot
AuxB1
ChRC5
% Cell growth
PSC833
F
12.5 µM AMA
AuxB1
ChRC5
% Cell growth
PSC833
G
AuxB1
CHRC5
% Cell growth
VRP: 3.125 µM
Rot: 1 nM
AMA: 1 nM

Figure 6. Effect of rotenone and Antimycin A on AuxB1 and CH^RC5 cell survival. Cell growth with or without drugs was assessed by MTT assay. In panel A and B AuxB1 and CH^RC5 cells were incubated with drugs (rotenone and antimycin A, respectively) for 24 hrs. In panel C and D cell growth as evaluated by MTT after siRNA transfection and 72 hrs incubation with 1 μM rotenone or 10 μM antimycin A, respectively B1; AuxB1, C5; CH^RC5, Mock; Mock siRNA transfected and si P-gp; P-gp siRNA transfected. Panel E and F show cell growth up to 72hrs exposure to rotenone or antimycin A with or without pretreatment with PSC833. Panel G shows the cell growth after 72 hrs treatment with a combination of 5 μM Verapamil and with or without 1 nM rotenone or 1 nM antimycin A. Error bars represent standard deviation of at least one representative experiment done in triplicate.

μM) or antimycin A (10 μM). While there was no effect on parental cells, the CH^RC5 siRNA treated cells were rescued almost up to the parental level of cell growth with both compounds. As expected, the control siRNA Mock transfected cells did not lead to any off target detectable effects. To confirm that the ATPase activity of P-gp was responsible for this collateral sensitivity to ETC inhibitors, we pretreated the cells with PSC833. PSC833 is a cyclosporin analog known to inhibit P-gp. In our previous paper we used PSC833 to successfully inhibit collateral sensitivity to verapamil and correlated this with inhibition of ATPase stimulation (7). Once again as shown in figure 6 E and F, the collateral sensitivity is rescued from both 0.1 μM rotenone or 12.5 μM animycin A up to the parental level. Having shown the collateral sensitivity to the ETC inhibitors, it was of interest to see the impact of these compounds on the collateral sensitivity when put together along with verapamil. We then used a non toxic concentration of rotenone (1 nM) that we combined with 5 μM verapamil, a concentration eliciting a strong collateral sensitivity. The same process was undertaken with Antimycin A (AMA) (1 nM) and a verapamil combination. Figures 6 G shows that the verapamil toxicity was significantly enhance by the presence of the ETC inhibitors (from 61 to 25 % cell growth for

rotenone and from 61 to 10 % cell growth for AMA) and this specifically for the CH^RC5 cells. The increase of toxicity using non toxic concentration of one compound suggests synergy between the two compounds.

Synergy of Verapamil with H_2O_2.

We initially hypothesized that P-gp ATPase activation was leading to higher energy demand, increasing ETC activity leading to induction of toxic ROS. Since rotenone and antimycin A were also leading to collateral sensitivity and synergy with Vrp as shown in figure 6, and these two compounds were also shown to induce ROS (228), it was of interest to determine if the same phenomenon would occur directly using ROS. We therefore combined the Vrp with H_2O_2. The combination of non toxic concentration of H_2O_2 (50 µM) and 5 µM verapamil as seen in figure 7 leads to a significant increase of verapamil toxicity. This result implies the presence of synergy between H_2O_2 + Vrp and suggest that verapamil toxicity is involving ROS production.

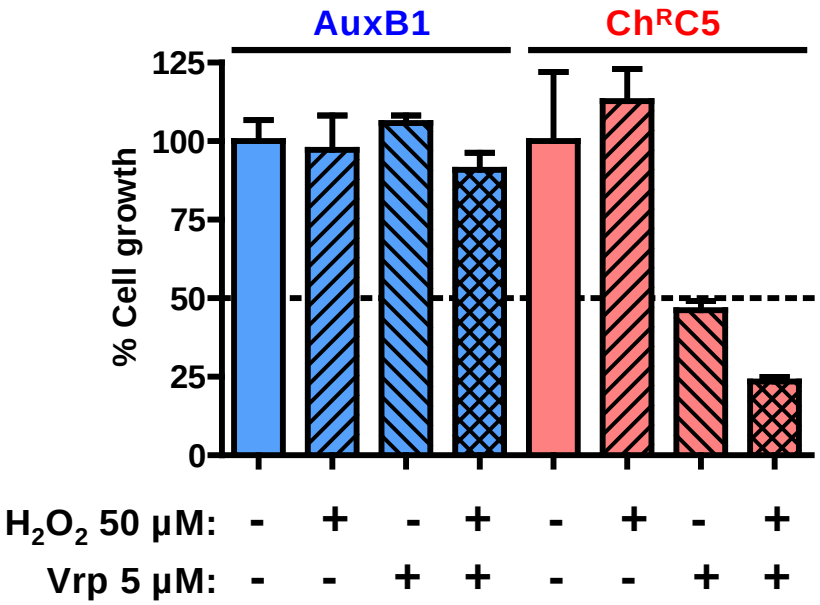

Figure 7. Effect of H_2O_2 on CH^RC5 and synergy with verapamil. Cell growth of CH^RC5 and AuxB1 evaluated by MTT assay. The % cell growth evaluated by MTT assay of CH^RC5 and AuxB1 submitted to 5 μM Verapamil (Vrp) for 72 hrs, with or without a pretreatment of 50 μM H_2O_2. Error bars represent standard deviation of a least one representative experiment done in triplicate.

DISCUSSION

The initial selection of CH^RC5 cells was followed by cloning (52), although previous work by our and other groups clearly showed that CH^RC5 shows this pattern of sensitivity. The clonal nature of CH^RC5 cells remains questioned, indeed the shape suggest the possibility of a mixed population eliciting different level of sensitivity. The fact that CH^RB30 (selected from CH^RC5 with higher resistance to colchicine) shows the same pattern does not discredit the possibility that the initial selection of CH^RC5 had mixed populations. If such is the case, the bell shape ATPase activation pattern would not explain the two step sensitivity. After recloning of the CH^RC5 cells in figure 1, the biphasic curve still appears, clearly showing that this effect is not due to a mixed population.

The results of P-gp1 down-regulation following transfection of CH^RC5 cells with siRNA are interesting, in light of earlier reports of P-gp1 half-life (229,230). Indeed, these results are consistent with a P-gp1 half that which is ~14 to 17 h. Hence, to the observed reduction in P-gp1 levels 24h after transfection further confirms the half-live of P-gp1 in these MDR-tumor cells (229,230). Moreover, the observed down-regulation of hamster P-gp1 was more pronounced than seen earlier with human Pgp1 using the same approach (226). Regardless, the results in figure 2 represent the first demonstration of down-regulation of hamster P-gp1 with siRNA in CHO drug-resistant cells.

The reversal of CH^RC5 P-gp1-siRNA transfected cells to verapamil collateral sensitivity are in contrast with the results seen earlier in figure 3, where down-regulation of P-gp1 expression caused a relatively weaker reversal in paclitaxel or doxorubicin, suggesting that the remaining levels of P-gp1 in cells was sufficient to confer drug resistance but not collateral sensitivity to verapamil. Based on these results it can be argued that a certain threshold level of P-gp1 expression is required (in the background of other cellular changes) to display verapamil collateral sensitivity. Indeed, these results may explain why not all P-gp1-expressing drug-resistant cells display verapamil collateral sensitivity.

The three fold basal plasma membrane ATPase activity of the CH^RC5 compared to the parental AuxB1 shown in figure 5 A leaves these cells in a delicate position facing ATP depletion as shown in figure 6 by the hypersensitivity to rotenone and Antimycin A. Furthermore figure 6 C to F proves this hypersensitivity to be due to the presence and activity of P-gp1 since it is reversed by P-gp1 knock down and chemical inhibition. The 2.5-fold stimulation of P-gp ATPase activity by verapamil shown in figure 5 B put these already sensitive cells to ATP depletion in a situation where they can survive. The ATP depletion in the P-gp1 expressing cells solely by VRP might not be enough to results in a global ATP depletion in the cells. Indeed, others showed that to see a global reduction in ATP levels, those cells need to be pretreated with an ATP generation inhibitor such as NaN_3 in glucose free media (231). The addition of ATP synthesis inhibitors to the ATPase stimulator leading to global ATP level reduction is also coherent with the synergy observed between rotenone or antimycin A and verapamil seen in figure 6 G.

We initially proposed that collateral sensitivity is primarily due to mitochondrial production of ROS in response to higher ATP turnover caused by activation of P-gp1 ATPase (7). To test this latter proposal, it was of interest to evaluate the survival of P-gp1-expressing cells treated with known ETC ROS generator alone and together with verapamil. The mitochondrial complex I and III are two of three major sites of electron transfer and consequently ROS production. Electrons that escape or leak from the ETC react with molecular oxygen to form superoxide radical ($O_2\cdot^-$) (232,233). rotenone and antimycin A are well characterized complex I and complex III inhibitors respectively, shown to increase ROS levels due to e^- release at their respective sites in the ETC (234,235). The combination of rotenone or antimycin A with verapamil showed a strong increase in collateral sensitivity in CH^RC5 cells by contrast to AuxB1 cells (figure 6) possibly due to a much greater ATP consumption and higher levels of resting total ATPase (figure 5). This increased demand for ATP is consistent with higher levels of P-gp1 in CH^RC5 cells. The synergetic toxicity observed between P-gp1 ATPase stimulator and ETC inhibitors implicates the

mitochondrial ETC and increase ROS production in the mechanism of collateral sensitivity. These results support our hypothesis that verapamil induced collateral sensitivity in P-gp1 positive cells due to increased ROS leading to oxidative cell death (7). This was also confirmed by the presence of synergy between verapamil and H_2O_2 in the CH^RC5 (figure 7).

In this report we confirm that the amount of P-gp is crucial in order to obtain collateral sensitivity to ATPase stimulator. Indeed, a fast reversal of hypersensitivity is observed in partial P-gp knock down cells while a more important mdr is still observed. Also, in our previous paper we show that the AuxB30, selected along with the CH^RC5 but expressing more P-gp, show a higher level of collateral sensitivity (7).

This suggests that the 2.5-fold increase in consumption of ATP in the presence of verapamil is not enough if there are only a few P-gp. This goes along with what was previously suggested by others, where a minimal threshold of P-gp expression is require to observe collateral sensitivity (225). This report was also suggesting that the genetic background is not related to the collateral sensitivity. Furthermore, emphasizing P-gp expression as a single requirement for collaterals sensitivity, the single transfection of mdr1 gene in NIH 3T3 cells leads to a collateral sensitivity to verapamil (95).

We discuss earlier that global ATP levels does not decrease with the addition of verapamil. If the ATP consumption does not reflect at the global cellular level, it can still reflect at the plasma membrane proximity. If such is the case, it is more than probable that other essential ATP requiring proteins at the plasma membrane level would not function properly. Of the 80% of oxygen consumption coupled to ATP synthesis, approximately 25-30% is used by protein synthesis, 19-28% by the Na^+-K^+-ATPase, 4-8% by the Ca^{2+}-ATPase, 2-8% by the actinomyosin ATPase, 7-10% by gluconeogenesis, and 3% by ureagenesis, with mRNA synthesis and substrate cycling also making significant contributions (236). The ionic homeostasis maintained by the Na^+-K^+-ATPase is also critical for cell growth, differentiation, and cell survival. Although the toxic effects of blocking the Na^+-K^+-ATPase by ouabain and

other selective inhibitors have been known for years, the mechanism of action remained unclear. However, accumulating evidence now endorses a close relationship between ionic homeostasis and apoptosis, namely the regulation of apoptosis by K^+ homeostasis (237). As important, the Ca^{2+}-ATPase help maintain low intracellular Ca^{2+} levels. Furthermore, verapamil was already shown to be able to increase intracellular Ca^{2+} levels in MDR CHO cells showing that it was not blocking the calcium channel but suggesting that it somehow blocked Ca^{2+} extrusion (5). Increases in Ca^{2+} levels is a strong apoptosis trigger what cooperatively works with ROS through various processes (159). Therefore, the ATP decrease induced by Vrp would have a dual effect of stimulating ROS production and increasing Ca^{2+} levels. The two combined would work in concert for apoptosis stimulation. MDR CHO cells are known for overexpressing the Ca^{2+} sequesters protein sorcin (98). The abundant presence of this protein leaves these cells with high intracellular Ca^{2+} levels (5). This could also leave the CHRC5 cells with intrinsic sensitivity to Ca^{2+} increases. Cells without hypersensitivity to Ca^{2+} modulation are therefore less susceptible to verapamil collateral sensitivity. Supporting this hypothesis, we determined by qPCR that human P-gp expressing cells (Mcf7/Adr) show very small increases in sorcin expression when compare to CH^RC5 increase and thus these cells highly MDR cells are not collaterally sensitive to Vrp (data not shown).

In conclusion, using siRNA, this study provides a direct link between P-gp1 expression and collateral sensitivity to verapamil. Moreover, we show that P-gp1-expressing CH^RC5 cells display collateral sensitivity to ETC inhibitors and such inhibitors act synergistically with verapamil to induce collateral sensitivity in CH^RC5 cells. Although several other factors with regards to the mechanism of collateral sensitivity remain to be resolved, these results support our hypothesis that verapamil induced collateral sensitivity in P-gp1 positive cells due to increased ATP consumption at the membrane level leading to ROS toxicity.

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Connecting Statement 2

Chapter 3 included a study that focused on the molecular mechanism of collateral sensitivity of ABCB1 (P-gp) expressing hamster cells. Specifically, we confirmed the involvement of P-gp in this mechanism. We also confirm the implication of P-gp ATPase overactivation induced by Vrp. Finally, we linked the phenomenon to ETC mediated ROS induction of toxicity and suggest the Ca^{2+} accumulation in the apoptosis induction. Some steroid hormones such as Pro and DOC, stimulate P-gp ATPase and were shown to induce collateral sensitivity in ABCB1 (P-gp) expressing hamster cells. Due to their P-gp ATPase activation activity that they share with Vrp, it is assumed that their mechanism of collateral sensitivity induction would be similar. However, since the mechanism was never thoroughly studied, this has yet to be confirmed. Chapter 4 examines the mechanism of collateral sensitivity of hamster P-gp expressing cells $CH^{R}C5$. This study explores the mechanism of collateral sensitivity to Pro and DOC, showing the differences and similarities with the Vrp mechanism.

CHAPTER 4

Collateral sensitivity to steroids directly modulated by hamster and human P-glycoprotein (ABCB1), differences and similarities with verapamil

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(manuscript in preparation)

ABSTRACT

In tumour cells, overexpression of P-glycoprotein (P-gp1, ABCB1) induces multidrug resistance by ATP dependant drug efflux across the membrane. In parallel, while inducing resistance to various compounds, the elevated expression of P-gp1 leads to hypersensitivity, or collateral sensitivity to other compounds. We already demonstrated that verapamil (Vrp) and some hormonal steroids (Progesterone and Deoxycorticosterone or Pro and DOC) induce collateral sensitivity through specific P-gp1 ATPase over-activation in the $CH^{R}C5$ CHO cells (Biochemistry. 2003; 42:12163-73). Consistent with an increased oxidative phosphorylation demand, we recently showed synergistic effect of Vrp and electron transport chain inhibitors (Laberge RM and Georges E, accompanying manuscript). In the same report, through the reversal of increased sensitivity/collateral sensitivity by siRNA experiments, we also prove this phenomenon to be dependant of P-gp1. By elucidating the mechanism(s) underlying the collateral sensitivity to Pro and DOC, we demonstrate in this report the similarities and differences between the latter and Vrp. First, we show that the phenomenon is dependant on both the presence and activity of P-gp1, by siRNA knockdown and chemical inhibition with PSC833. We also show that Vrp is able to inhibit Pro and DOC collateral sensitivity. As seen with Vrp, Pro and DOC also synergized with ETC inhibitors. A major difference with Vrp mechanism is the ability of cyclodextrin (a membrane steroid depleting agent) to reverse the collateral sensitivity. Finally, P-gp specific collateral sensitivity in the selected breast cancer cell line Mcf7/Adr was successfully obtained with Pro and DOC. Together these results demonstrate the requirement of active P-gp for steroid hormones collateral sensitivity and also suggest the need for membrane cholesterols. Moreover, to our knowledge for the first time we show that the P-gp ATPase overstimulation mechanism of collateral sensitivity to be observed in human cell line.

INTRODUCTION

Drug resistance is the major cause of treatment failure in clinical oncology (21). Some tumours may be intrinsically resistant to chemotherapy prior the treatment, while others, initially sensitive to chemotherapy, can acquire resistance during the treatment. Furthermore, MDR may develop; this is a process that involves cross-resistance to a range of chemically unrelated agents with different cellular targets. Several possible mechanisms leading to MDR have been postulated, however, one of the most intensively studied mechanisms involves the overexpression of specific ABC proteins that mediate active drug efflux (21). Indeed, the expression of these proteins decreases the accumulation of the active compounds, therefore reducing its toxicity. Classically, MDR induced by ABC transporter expression is seen as acquired resistance at the tumour level. With the recent resurgence of CSC hypothesis, it appears this view of MDR will change again. Indeed, the cancer initiator cells with stem cell properties would overexpress ABC transporters, mainly P-gp and BCRP (27,28). This strongly argues for the important role for tumour initiator cells in propagating MDR and explaining the relapses (27). Therefore, ways of targeting P-gp expressing cells is more than necessary.

Collateral sensitivity is defined as the increase of sensitivity or hypersensitivity of a MDR cell line to a certain compound while showing resistance to others. In P-gp expressing cells, collateral sensitivity to the glycolysis inhibitor 2-Deoxyglucose, to the extrinsic apoptosis pathway inducer TRAIL and to NSC73306, a thiose-micarbazone, was observed (92-94). The first paper reporting collateral sensitivity arose almost at the same time when MDR cell line were selected and by the same group. In 1976, Victor Ling's group showed that their colchicines-resistant CHO cells were hypersensitive to steroid hormones and detergents (2). Apart from correlating the ATPase activation with the hypersensitivity to Vrp mechanism (7), this hypersensitivity to steroid hormones was never further investigated. Here, we provide more insight by demonstrating the similarities and differences with the Vrp mechanism that we recently elucidated (Laberge and Georges, accompanying

manuscript). We also show cell rescue with pretreatment by cyclodextrin suggesting the requirement of cholesterol for the active collateral sensitivity. Conversely to Vrp, the steroids collateral sensitivity phenomenon seems to only require P-gp and therefore was successfully find in MDR human cancer cells.

MATERIALS AND METHODS

Cell Culture and siRNA Transfection. AuxB1 and drug-resistant selected cell line (CH^RC5, a kind gift from Dr. V. Ling, at the BC Cancer Centre, British Columbia, Canada) and Mcf7 and drug-resistant selected cell line (Mcf7/Adr) were grown in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO_2 without or with 5 μg/ml colchicine (CH^RC5) or 1 μM doxorubicin (Mcf7/Adr). For siRNA transfection, siRNA duplex sequences corresponding to the Chinese Hamster mdr1 sequence [5'AGAGAAGAAACCAGUGGUC3' (sense) and 5'GACCACUGGUUUCUUCUCU3' (antisense)], to the Human mdr1 sequence [5'GGAAAAGAAACCAACUGUC3' (sense) and CCUUUUCUUUGGUUGACAG5' (antisense)] or negative control (Mock) siRNA duplex sequences [5'UAAAGAGUUACCCUAGUUUCA3' and 5'UGAAACUAGGGUAACUCUUUA3'] were synthesized by Invitrogen Life technology Inc. Cells in exponential phase at 50-60% confluency were transfected with 1 nmole siRNA complexed with lipofectamine 2000 (Invitrogen, Burlington, On, Canada) in serum-free α-MEM according to manufacturer's protocol, with some modification. Briefly, prior to transfection, cells were washed once with serum-free α-MEM and incubated with the transfection mix for 6 h. Cells were washed with serum-containing media and incubated with or without drugs for the rest of the experiment.

Cell Growth Assay. Cells transfected with siRNA sequences were seeded in triplicate into 96-well plates 48 h post-transfection. Following 24 h incubation, cells were exposed to increasing concentrations of drugs and allowed to grow for 72 h. For drug combinations, cells were preincubated 4 hours with PSC833, rotenone or antimycin A before adding steroids or Vrp. For drug combinations with cyclodextrin, cells were preincubated for 1 hour with cyclodextrin (in serum free media), then removed and the cells were washed prior to the addition of steroids or Vrp. The assay was developed by the addition of 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) dye to a final

concentration of 0.5mg/ml into each well and plates were incubated at 37°C for 4 h. The MTT crystals were dissolved with 200 µL of DMSO. Cell growth was determined from the absorbance values measured at 570 nm relative to control or untreated cells.

Protein Extraction and Immunodetection of hamster cells. siRNA transfected cells ($1X10^6$) were harvested every day for 6 days post-transfection. Cells were washed three times with PBS and lysed in 50 µl of lysis buffer (50 mM Tris-Cl pH 7.8, 150mM NaCl, 1% NP-40, containing 1% protease inhibitor cocktail). The cell lysate was centrifuged at 13,000 rpm for 20min and the supernatant fraction was stored at -20 until further use. For immunodetection by Western blots, 25 µg sample of cell lysate was resolved on Fairbanks gels (54) and resolved proteins were transferred onto a nitrocellulose membrane (223). The same nitrocellulose membrane was probed for P-gp1 and actin expression using a P-gp1-specific monoclonal antibody (265/F4, Abcam, Cambridge MA) and actin specific polyclonal antibody (20-33, Sigma, Oakville, On, Canada) at 1:250 (v/v) dilution, respectively. HRP-conjugated goat α-mouse or α-rabbit IgGs (BioRad, Hercules, CA) were used at 1:3000 v/v dilution as secondary antibodies. HRP detection was performed using Immobilon Western detection kit (Millipore, Billerica, MA) according to manufacturer instructions. For ELISA, 2 µg of cell lysate was used to coat the wells of 96-wells plates in 10mM ammonium bicarbonate buffer, pH 10, for 2 h at room temperature. The wells were washed three times with PBS and blocked with 1% BSA in PBS for 2 h. The assay was developed with the same primary (265/F4 mAb or α-actin pAb), and secondary (HRP-conjugated goat α-mouse or α-rabbit IgG) antibodies diluted in blocking buffer. P-gp1 expression was determined by absorbance at 450 nm relative to actin levels and AuxB1 cell lysate used as background for P-gp1.

Plasma Membrane Preparation and ATPase Assay. AuxB1 and CH^RC5 cells were washed with PBS and resuspended in 10 mL hypotonic buffer

(10mM HEPES-Tris pH 7.4, 5 mM EDTA, 5 mM EGTA, and 2 mM dithiothreitol) containing protease inhibitors (2 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL pepstatin). Cells were ruptured with a glass homogeniser (Kontes, Vineland, NJ) and plasma membrane isolated by differential centrifugations, with a final centrifugation at 45000x*g* for 60 min (7). The latter membrane pellets were resuspended in 1 mL buffer using a 27-gauge syringe and stored at –80°C. P-gp1 ATPase activity was determined by quantifying the release of inorganic phosphate from ATP as previously described (7). P-gp1 specific ATPase activity was determine in the presence of mitochondrial, Ca^{2+} dependant and Na^{+} / K^{+} dependant ATPase inhibitor; 10 mM NaN_3, 4 mM EGTA and 2 mM Ouabain. Each condition represents experiment done three independent times with six replicates.

Intact cell ELISA for human P-gp detection. siRNA transfected Mcf7, Mcf7/Adr and respective mock control cells were harvested daily during a course of 5-day experimental period. 10,000 cells were seeded in a 96-well plate and allowed to adhere for 4 hours. After removing the media, the cells were incubated with 30µl 1:100 MRK-16 (P-gp) primary antibody or anti-GSH (control) for 30 min at room temperature with gentle agitation. After washing three times with the media, cells were incubated with 30µl HRP-conjugated Goat anti-mouse IgG (1:2000, BIORAD) for 30 min at room temperature with gentle agitation. The cells were then washed thrice with PBS and were reacted with the detecting solution-TMB substrate (3,3',5,5' tetramethylbenzidine, SIGMA) for 10-15 min at room temperature in dark. The reaction was quenched by adding 50 µl of 2N H_2SO_4 and the optical density was measured at 450 nm using a microplate reader (Dynatech MR5000).

Statistics. All graph and statistics were performed using Graphpad prism version 4. All statistics represent the student t test.

RESULTS

Reversal of collateral sensitivity by P-gp knockdown. To determine if the P-gp ATPase stimulation observed with Pro and DOC could be translated in collateral sensitivity, we used the same CH^RC5 and AuxB1 cells treated with siRNA against P-gp (Laberge R.M. and Georges E., accompanying paper) and subjected them to increasing concentration of these compounds. The results in figure 1 A and C show that CH^RC5 cells are collaterally sensitive to both Pro and DOC, relative to AuxB1 cells with significantly different IC_{50}. More importantly, transfection of Pgp1 specific siRNA in CH^RC5 cells versus mock siRNA transfected or untransfected results in a large significant reversal of Pro and DOC collateral sensitivity of CH^RC5 cells. To verify if the collateral sensitivity reversal was not due to off- target effect of the siRNA, we performed the transfection on the parental AuxB1 cells as well. As expected, figure 1 B and D show that transfection did not lead to any change in sensitivity to Pro or DOC. These results, like as for Vrp, demonstrate that the collateral sensitivity mechanism is P-gp1 dependent.

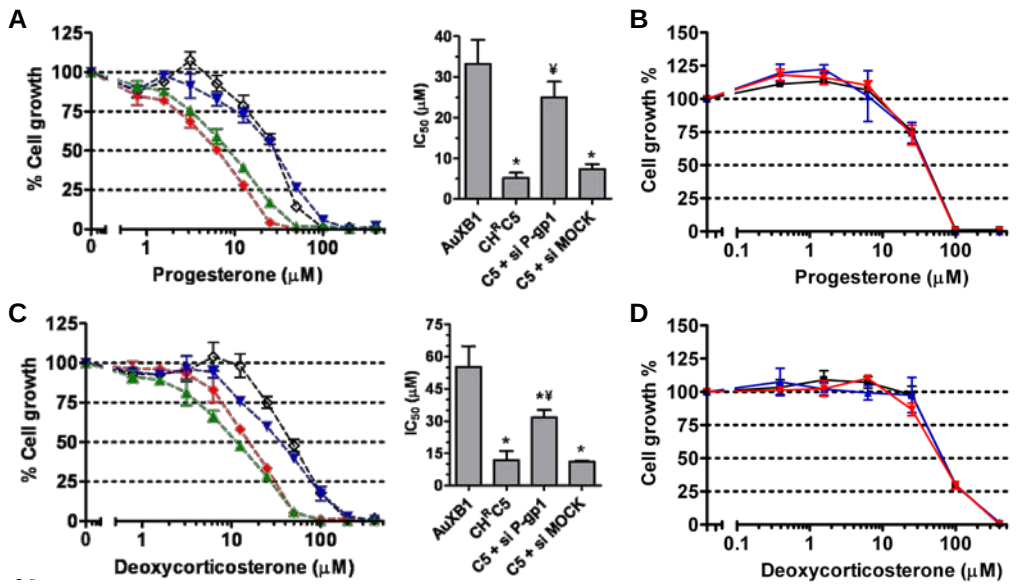

Figure 1. Effect of progesterone and deoxycorticosterone on the survival of tumor cells with or without P-gp1 expression. Cell growth was assessed by MTT assay. Parental or drug-sensitive cells (AuxB1 cells; open diamond), P-

gp-expressing tumor cells (CH^RC5 cells; filled diamond), P-gp1-siRNA transfected CH^RC5 panel A and C AuxB1 panel B and D cells (inverted triangle) and Mock siRNA transfected CH^RC5 panel A and C AuxB1 panel B and D cells (triangle), were grown in the presence of increasing concentrations of Progesterone (panel A) or deoxycorticosterone (panel C) for 72 h. Error bars represent standard deviation from at two different experiments done in triplicate. * and ¥, $P < 0.01$, statistically significant difference compared to AuxB1 (*) or CH^RC5 (¥).

Chemical Reversal of MDR and Collateral Sensitivity. We have previously demonstrated that the cyclosporine analogue and P-gp ATPase inhibitor, PSC833, is able to reverse collateral sensitivity to Vrp (Laberge R.M. and Georges E., accompanying paper). Having confirmed in figure 1 that the collateral sensitivity could be reversed by P-gp knock down, it was of interest to see if the ATPase inhibitor PSC833 was able to reverse collateral sensitivity to Pro and DOC. As PSC833, being a cyclosporine analog, could possibly reverse apoptosis, we first wanted to control for this effect in the AuxB1 and CH^RC5 using the non P-gp1 substrate and known apoptosis inducer drug Cis-platinum. As we see in figure 2 A, the toxicity induced by 7 µM Cis-platinum was not reversed by pretreatment with 2 µM PSC833 in either AuxB1 or CH^RC5. This control confirms that PSC833 would not inhibit apoptosis,which would have led to false interpretation of P-gp associated toxicity reversal. To validate the ability of PSC833 to inhibit P-gp, we subjected PSC833 pre-treated cells to 10 µM concentration of the known P-gp subtrate doxorubicin (figure 2 B). As expected, this pretreatment with PSC833 did not change toxicity (0 % cell growth) in the AuxB1, where as the resistance seen in the CH^RC5 was totally abolished (from 80 % to 0 % cell growth). Finally, in figure 2 C and D we observed that both Pro and DOC toxicity can be rescued by the addition of PSC833 in the CH^RC5 (from 30 % to 105 % and from 45 % to 100% cell growth, respectively). As seen in figure 2, the concentrations used (20 and 37.5 µM of Pro and DOC, respectively) were not toxic for the AuxB1 cells and the

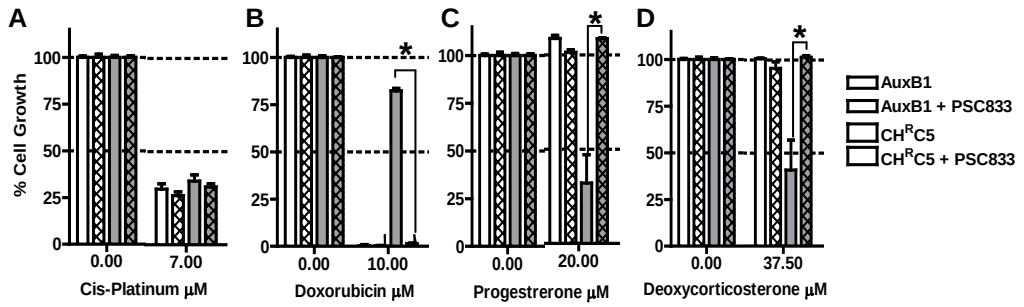

Figure 2. Effect of PSC833 on drug sensitivity to AuxB1 and CH^RC5. Cell growth with or without drugs was assessed by MTT assay. Parental (AuxB1) and resistant (CH^RC5) cells were grown in the presence of selected drug concentration [Cis-Platinum (panel A), Doxorubicin (panel B), Progesterone (panel C) or Doxorubicin (panel D)] for 72 hrs. When used, 2 µM PSC833 was added 4 hrs prior to the addition of the other compounds. Error bars represent standard deviation from one representative of at least two experiments done in triplicate. * indicates, $P < 0.01$, statistically significantly different.

PSC833 had no effect on them either. Together this figure shows the importance of P-gp ATPase activity for collateral sensitivity to Pro and DOC.

P-gp ATPase Activity Stimulation. We have previously shown that P-gp1 ATPase stimulation by Vrp correlates with the concentration eliciting collateral sensitivity in the CH^RC5 cells (7). Steroids, such as Pro and DOC, are also known to stimulate P-gp ATPase activity. This concentration dependent profile is shown in figure 3. Pro stimulates two fold the basal ATPase at 100 µM and a ½ max at 20 µM, consistant with previous findings (7,82,83). DOC stimulated the ATPase activity 1.6-fold at 50 µM max and ½ max at 12.5 µM.

Dose Dependant Collateral Sensitivity Inhibition by Verapamil. It is known that Vrp and steroid hormones interact with P-gp to stimulate its ATPase activity. Nonetheless, the "how" and "where" this interaction is occuring remains unknown. Therefore, it was of interest to find

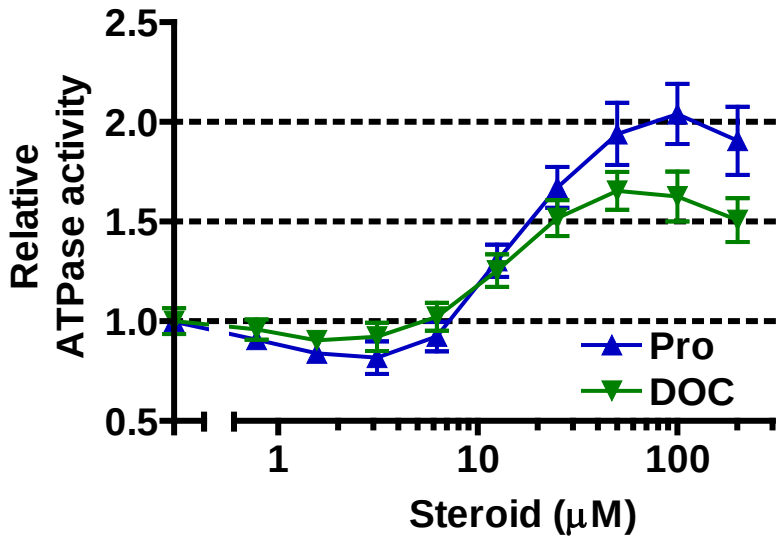

Figure 3. Effects of progesterone and deoxycorticosterone on P-gp1 ATPase activity. P-gp1 specific ATPase was measured using purified CH^RC5 plasma membranes exposed to increasing concentrations of progesterone (triangle) or deoxycorticosterone (inverted triangle). ATPase activity is depicted in the graph relative to the non-stimulated activity. Error bars represent standard deviation from at least two representative experiments done in triplicate.

out what would result from their combination. We mixed active concentrations (i.e. inducing collateral sensitivity) of Pro or DOC with increasing concentrations of Vrp. As can be seen in figure 4 A and C, none of the combinations induced changes in cell survival in the control parental AuxB1 cell line. Figure 4 B shows that 20 μM of Pro led to 35 % cell growth in the CH^RC5 cells. This reduction of cell growth is partially reversed with the addition of 0.4 μM Vrp. Furthermore, increasing the concentration to 2 and to 10 μM led to the reversal of Pro induced cell growth inhibition up to the level of vrp toxicity (70 % cell growth at 10 μM). This suggests a competition for the two compounds but also a greater affinity for Vrp. Similar results were obtained with DOC (figure 4 D). However, in this case, significant rescue with Vrp was achieved at a concentration of 10 μM and not at lower concentrations. This

once again suggests a competition for the same binding site but with a higher affinity for DOC than Pro. Alternatively, the apparent competition could be the results of allosteric modulation.

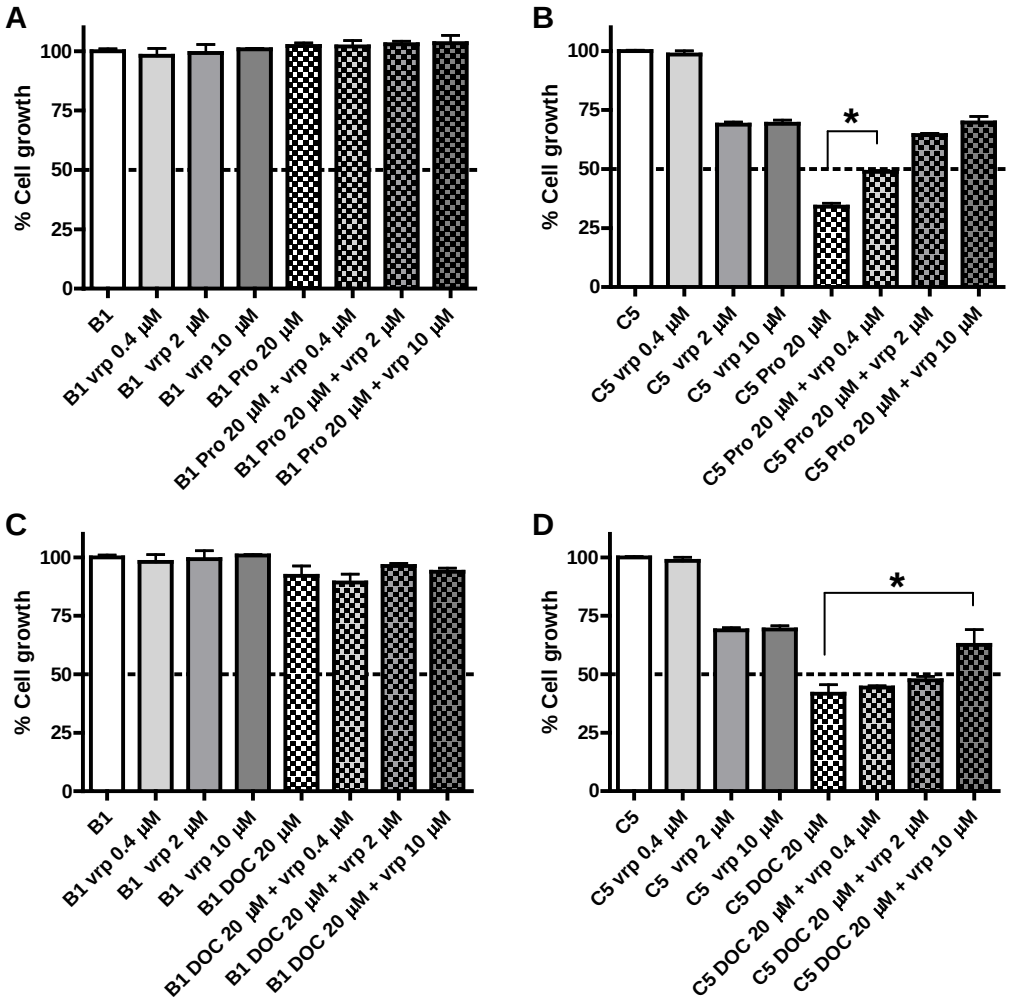

Figure 4. Effect of verapamil on steroid induced collateral sensitivity. Cell growth with or without drug was assessed by MTT assay. AuxB1 (B1) (panel A and C) and $CH^{R}C5$ (C5) (panel B and D) cells were incubated in the presence of increasing concentrations of verapamil (vrp) with or without simultaneous addition of 20 µM of progesterone (Pro) (panel A and B) or deoxycorticosterone (DOC) (panel C and D) for 72 hrs. Error bars represent standard deviation of one representative experiment repeated at least twice done in triplicate. * indicates, $P < 0.01$, statistically significantly different.

Synergy with Electron Transport Chain Inhibitors. We showed previously that Vrp (P-gp ATPase stimulator) synergizes with the well known ETC inhibitors Rotenone and Animycin A, in terms of toxicity (Laberge RM and Georges E, accompanying manuscript). The results in figure 5 show that such a phenomenon also occurs with Pro and DOC. Pro (7.5 μM) or DOC (15 μM), in the presence of rotenone, has reduced the cell survival in CH^RC5 (figure 5B) but not in AuxB1 (figure 5A), as compared to the non-treated cells. Indeed figure 5 B shows that both the Pro and the DOC lead to a significant toxicity (80 % and 67 % cell growth respectively). This toxicity is amplified significantly by the presence of the non-toxic concentration of Rotenone (46 % and 34 % cell growth respectively). Similar results were obtained with 10 nM AntimycinA (figure 5C & D). Here the intrinsic toxicity of Pro and DOC is amplified from 79 % to 15 % cell growth, and from 72 % to 11 % cell growth respectively. The addition of Antimycin A alone leads to a significant reduction of cell growth as well (80 % cell growth). Since the combination leads to a cell growth diminution more important than the sum of their individual ones, one can conclude that they synergize. In the AuxB1, these latter combinations did not lead to any increase of toxicity. Indeed, figure 5 C shows that no difference of cell growth is observed when either Pro or DOC is added along with antimycin A Figure. Such as in CH^RC5 antimycin A leads to a small but significant reduction of cell growth (85 %).

Cell rescue with membrane cholesterol depleting agent cyclodextrin. To determine if membrane cholesterol could have any effect on the collateral sensitivity induced by Pro or DOC, we pretreated the cells with the known membrane cholesterol depleting agent methyl-β-cyclodextrin. As shown in figure 6A, incubation of AuxB1 cells with 30 μM Pro or 60 μM DOC led to a significant toxicity (65 % and 60 % cell growth respectively), while the other concentrations (7.5 μM and 15 μM) and drugs (5 μM Vrp) did not reduce the

cell growth. This toxicity was completely reversed by the pretreatment with cyclodextrin (121 % and 107 % cell growth in combination with Pro or DOC,

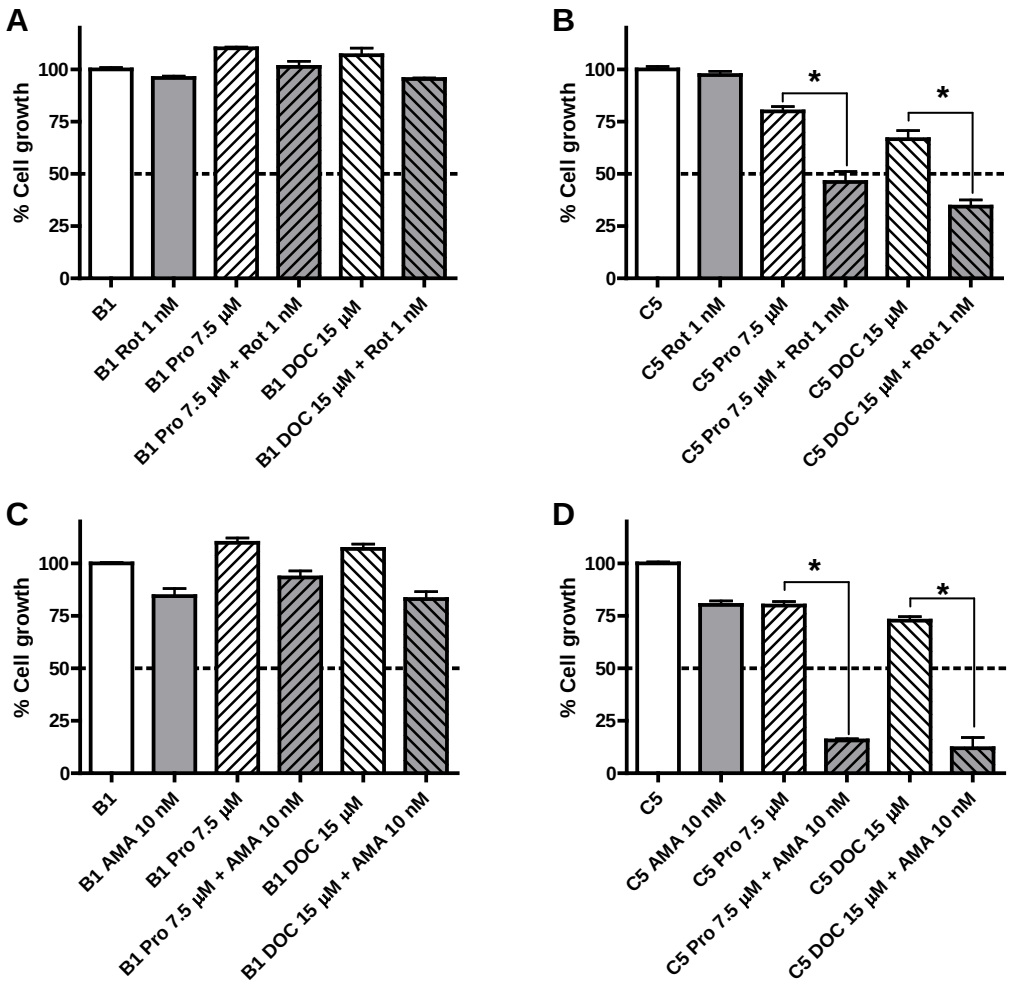

Figure 5. Effect of rotenone and antimycin A on collateral sensitivity to progesterone and deoxycorticosterone. Cell growth with or without drug was assessed by MTT assay. AuxB1 (B1) (panel A and C) and $CH^{R}C5$ (C5) (panel B and D) cells were pre-incubated or not with 1 nM rotenone (Rot) (panel A and B) or with 10 nM antimycin A (AMA) (panel C and D) in the presence or absence of either 7.5 µM progesterone (Pro) or 15 µM deoxycorticosterone (DOC) for 72 hrs. Error bars represent standard deviation of one representative experiment repeated at least twice done in triplicate. * indicates, $P < 0.01$, statistically significantly different.

respectively). Figure 6B shows the results for CH^RC5 cells. Here in we see that the toxicity induced by 7.5 µM Pro could be reversed by the cyclodextrin pretreatment (from 24 % to 93 % cell growth). The toxicity of 30 µM Pro is

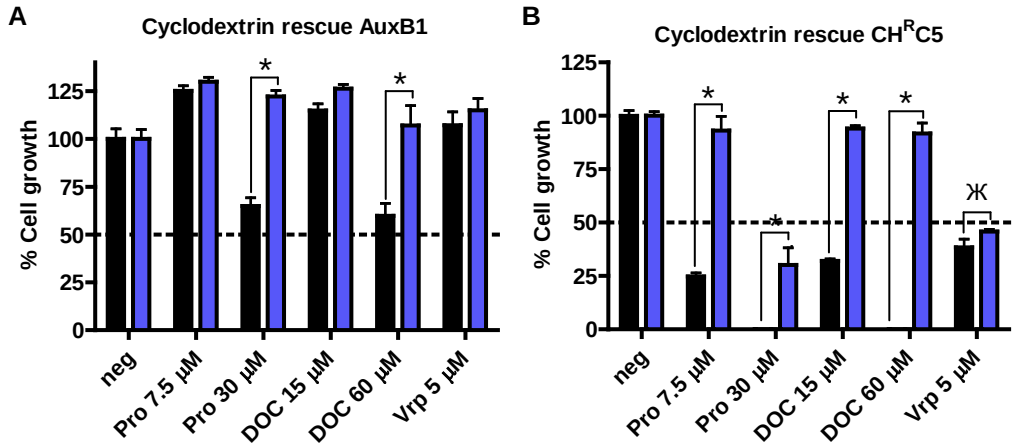

Figure 6. Effect of cyclodextrin on CHO cell survival. Cell growth after 1 hr preincubation with or without 10 mM cyclodextrin combined with or without various concentrations of either progesterone (Pro), deoxycorticosterone (DOC) or verapamil (Vrp) for 72 hrs. Cell growth was assessed by MTT assay. AuxB1 (panel A) cells and CH^RC5 (panel B) cells were incubated without drugs. Error bars represent standard deviation of one representative experiment repeated at least twice done in triplicate. * indicates, $P < 0.01$, statistically significantly different, Ж indicates, $P > 0.05$, statistically non-significantly different.

partially reversed (from 0 % to 30 % cell growth). Similar results were also obtained with DOC at 15 µM and at 60 µM (from 33 % to 94 % and from 0 % to 92 % cell growth, respectively). If cyclodextrin was able to reverse the toxicity of the two steroids, this is not the case for Vrp. Indeed, at 5 µM Vrp leads to a cell growth of 39 % and the addition of cyclodextrin raises non-significantly this percentage to 45.

P-gp Expression after MDR1 mRNA Treatment in Mcf7 cells. Having shown that the collateral sensitivity in CH^RC5 could be reversed through P-gp1 targeted siRNA (figure 1), here (figure 7), we have now down regulated the P-

gp in the human breast carcinoma expressing P-gp cells Mcf7/Adr. To do so, we used the previously published siRNA sequences for human P-gp1 (226). As shown in figure 7, the expression of surface P-gp was significantly reduced. Indeed, as

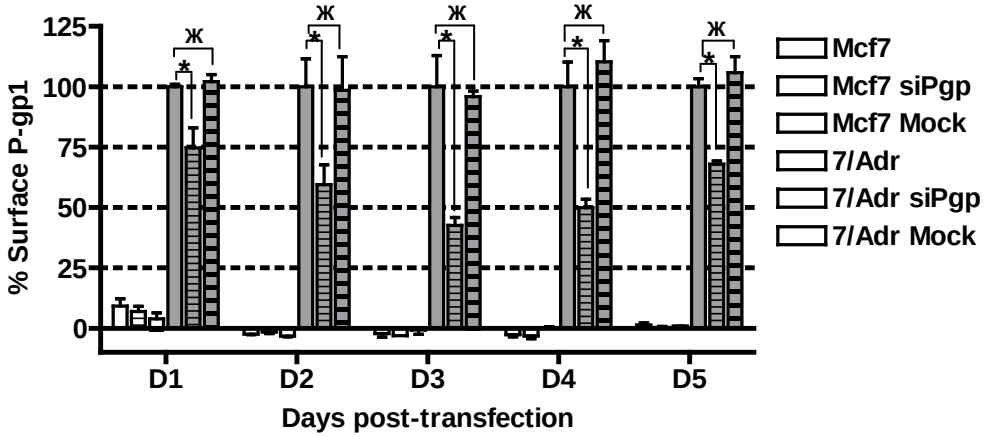

Figure 7. Down-regulation of P-gp1 expression with siRNA in human breast carcinomas. Relative levels of P-gp1 expression was measured in Mcf71 and Mcf7/Adr transected or not with Mock siRNA (Mock) or with P-gp1 siRNA (siPgp). The relative surface P-gp detected by intact ELISA is expressed in function of non transfected cells. Error bars represents Standard deviation of one representative experiment done in triplicate. * indicates, $P < 0.01$, statistically significant different, Ж indicates, $P > 0.05$, statistically non-significantly different.

detected by intact cell ELISA as early as day-1 post-trasnfection, the cell surface protein expression is reduced to 75 % relative to the level of the non-transfected control (Mcf7/Adr). The reduction of expression, peaks after three days post-transfection with 42 % relative to control. The levels for the other time points are 60 %, 50 % and 67 % for days 2, 4 and 5, respectively. Transfection with mock siRNA had no significant effect on P-gp levels. Finally, non significant detectable levels of P-gp was found in the parental Mcf7 and no effect after transfections. Detection was also performed using FACS and the

same MRK16 antibody to validate the method with similar results (data not shown).

P-gp Knock Down and its effect on collateral sensitivity. To confirm that the decrease in P-gp1 expression is reflected phenotypically, we examined the sensitivity of untransfected, mock and P-gp siRNA transfected Mcf7/Adr cells to doxorubicin, an anticancer drug that is a known substrate for P-gp1. Figure 8 A shows the growth of untransfected, mock and P-gp1 siRNA transfected Mcf7/Adr cells, as well as the parental Mcf7 in the presence of increasing concentrations of doxorubicin. Results from Mcf7 transfected cells were voluntary omitted from the graphs for clarity; there was no significant impact of these on the toxicity. As expected, a partial reversal of drug resistance was seen in the P-gp siRNA transfected cells compared to the non-transfected and the Mock transfected cells (figure 8A). The same reversal can be observed chemically with the P-gp inhibitor PSC833. Indeed, figure 8 B shows that the treatment in the Mcf7/adr bring the toxicity of 10 μM doxorubicin from 89 % to 6 % cell growth. To confirm the specificity of the siRNA, the cells were treated with the non P-gp substrate chemotherapy agent Cis-Platinum. Figure 8 C shows that the P-gp siRNA treatment did not have an impact on toxicity. In this figure we can also see a small difference in the shape of the curve between the resistance and the parental and around the IC50, the mock transfected cells are closer to the parental. If the P-gp knock down did not lead to any survival changes, the chemical inhibition did not either, as showed in panel D. These results validate the use of both the P-gp siRNA and the PSC833 to investigate the relation of P-gp and the collateral sensitivity in these human cell lines. In figure 8 E, cells were incubated with increased concentrations of Pro. The results show collateral sensitivity in the Mcf7/Adr with IC50s of 27 μM and 92 μM for Mcf7/Adr and the parental Mcf7, respectively. To our knowledge, this is the first demonstration of collateral sensitivity to steroid hormones in a human cell line. Confirming the necessity of P-gp for the mechanism, P-gp siRNA transfection reversed partially the collateral sensitivity (IC50 of 52 μM),

while mock did not have an effect (IC50 of 29 µM). Chemically, the same reversal effect can be seen with 50 µM Pro. As shown in panel F, % cell growth from 50 to 93 in mcf7/Adr when they are pretreated with PSC833. As shown in figure 8 G, collateral sensitivity is also found for DOC with an IC50 of 41 µM and 180 µM for Mcf7 and Mcf7/Adr, respectively. Once again, transfection with

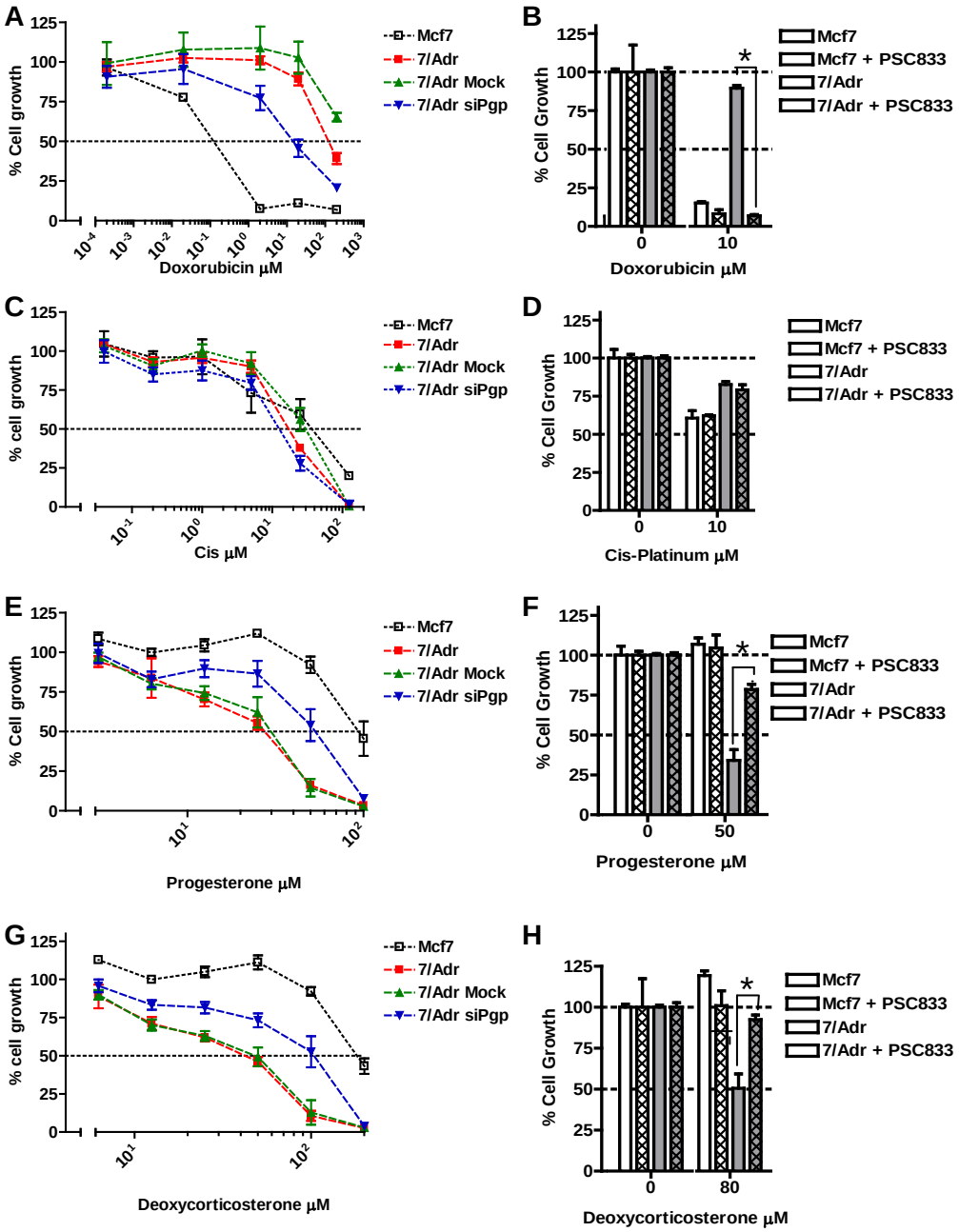

Figure 8. Effect of Doxorubicin, Cis-platinum, Progesterone or Deoxycorticosterone on the survival of tumor cells with or without P-gp1 expression. Cell growth with or without drugs was assessed by MTT assay. Parental or drug-sensitive cells; Mcf7 (7) , P-gp expressing tumor cells; Mcf7/Adr (7/Adr), P-gp1-siRNA transfected cells; Mcf7/Adr (7/Adr siPgp) and Mock siRNA transfected cells; Mcf7/Adr (7/Adr Mock), were grown in the presence of increasing concentrations of Doxorubicin (panel A), Cis-Platinum (Cis) (panel C), Progesterone (panel E) or Deoxycorticosterone (panel G) for 72 h. Alternatively, non-transfected cells were pretreated 4 hours with 2 μM PSC 833 prior to the addition of the other mentioned drugs for 72 hrs (panel B, D, F and H). Error bars represent standard deviation from at least one representative experiment done in triplicate. * indicates, $P < 0.01$, statistically significantly different.

siRNA against P-gp reverses partially the effect (IC50 of 102 μM), while mock had no effect (IC50 of 46 μM). Finally, in panel H we can see that the reversal is also achieved chemically. Indeed, 80 μM DOC leads to 50 % cell growth, while it is 93 % when pretreated with PSC833.

DISCUSSION

P-gp1, along with BCRP, was shown to be highly expressed in cancer stem cells, thus these cancer initiator cells potentially elicit intrinsic MDR, emphasizing the importance of development of effective strategies to target ABC transporter expressing cells. In the accompanying paper, we demonstrated that Vrp induces collateral sensitivity in $CH^{R}C5$ cells by over-activating P-gp1 ATPase, thereby resulting in ROS production through ETC increased demand (Laberge RM and Georges E). Although being a prerequisite, as shown by P-gp1 knock down, this mechanism does not appear to be the only essential factor. The higher calcium level induced by sorcin overexpression could also be involved. In an attempt to further unravel the mechanism of collateral sensitivity to P-gp ATPase stimulating agent, we studied the effect of the steroid hormones Pro and DOC. In this manuscript, we demonstrate that, as for Vrp, the mechanism of collateral sensitivity of the steroids hormones require the presence of an active P-gp and a surcharge of ETC. Although, conversely to Vrp, the collateral sensitivity to steroid hormones seems to require cholesterol at the membrane. Another important difference is that the collateral sensitivity could be observed in human P-gp expressing cells.

In this manuscript we have shown that both Pro and DOC induces collateral sensitivity in $CH^{R}C5$ cells (figure 1). This phenomenon could be reversed at least partially through P-gp knockdown, as well as through inhibition of ATPase activity with PDC833 (figure 2). Together, these results demonstrate the necessity of P-gp presence accompanied with its ATPase capability, so far being consistent with Vrp mechanism of collateral sensitivity (Laberge RM and Georges E, accompanying manuscript) (7). P-gp is highly abundant at the plasma membrane level, and this could change the membrane fluidity. Therefore, chemical inhibition of P-gp with PSC833 is important, as it rules out the possibility of a simple physical effect due to high abundance of protein at the membrane. It is interesting to notice here as well that the reversal of collateral sensitivity with siRNA against P-gp1using the same cells is more

important for the steroid hormones than it was with Vrp. This difference could be attributed to the importance of calcium modulation of Vrp while it is not a factor for the steroid hormones.

In figure 3 we show that, like Vrp, the activation of ATPase corresponds to sensitivity in the $CH^{R}C5$. This appears early in the ATPase activation. Indeed, for Pro the ½ max ATPase activation is obtained at 18 μM, while the IC50 is 5 μM. Equally, for DOC they are 25 μM and 12 μM, respectively. These results indicate that only a low ATPase activation is required for the apparition of toxicity and suggest that other factors could be involved as well. We suggested the ATPase activation to lead to toxic ROS formation. We used known ETC inhibitors (rotenone and antimycin A) to induce ROS to demonstrate a synergy with the collateral sensitivity agents (234,235). Like vrp, Pro and DOC also lead to synergy as shown in figure 5 suggesting the involvement of ROS in the collateral sensitivity mechanism.

It has been suggested that Vrp and steroid hormones could synergize in their ATPase activity (9,83). In addition to these findings, another report also concludes that these drugs compete for the same binding site in function of the concentration, and that there would be several binding sites with possible allosteric effects leading to competitive, non-competitive or cooperative effects (8). It was therefore of interest to find out if these combinations would be reflected in synergy or rescue at the toxicity level. As shown in Figure 4, Vrp rescued CHO cells from Pro or DOC collateral sensitivity. For Pro the rescue starts with as low as 0.4 μM Vrp while for DOC it starts at 10 μM. At least for Pro, (DOC was not previously described) these numbers reflect those already published for ATPase activity (8). This report shows inhibition of 20 μM Pro P-gp ATPase activation with Vrp starting at 0.4 μM and increasing gradually with increase in concentration. In the report, they also suggest at least for these concentrations an competitive inhibition or not but no additive effect once again consistent with ATPase assay (8).

We mentioned earlier the possibility of a parallel mechanism that would potentiate the effect of the primary ROS production mediated toxicity.

The results in figure 6 showing reversal of collateral sensitivity with the membrane cholesterol depleting agent (cyclodextrin) follow this hypothesis. Reports suggest the requirement of cholesterol for proper P-gp ATPase activity, this could therefore explained the rescue. Indeed, it has been shown that cholesterol stimulates basal (i.e. without any drugs) ATPase activity (77) (78). Moreover, when no cholesterol is added to P-gp containing proteoliposomes, the stimulated and basal ATPase activity is completely abolished (84). Although the Pro and DOC collateral sensitivity reversal by cyclodextrin would follow this hypothesis, the non-reversal observed with vrp suggests something else. Moreover, cyclodextrin is able to reverse the non-P-gp dependant cell death (in AuxB1) induced by Pro and DOC. Finally, the use of cyclodextrin typically results only in partial diminution of cholesterol levels, which is likely insufficient for total inhibition (238). Together, these results suggest that Pro and DOC induced cell death is potentiated by the presence of cholesterol in the membrane by an undefined mechanism. The presence of P-gp would amplify this toxicity due to the ATP consumption and the resulting ROS formation.

Human cells also contain cholesterol in their membranes, therefore the collateral sensitivity should also be observed in the P-gp expressing cells. In figure 8, to our knowledge for the first time, collateral sensitivity to steroid hormones can be seen in human cells. Moreover, a successful depletion using siRNA against the human P-gp (figure 7), led to reversal of collateral sensitivity. Finally, using the P-gp ATPase inhibitor, rescue was also observed. More experiments are underway to confirm the universality of the mechanism. It is interesting to note here that vrp failed to induce collateral sensitivity to human cell lines (data not shown). This emphasizes that ATPase activation alone is not enough for induction of hypersensitivity.

Together, the results suggest an expandable mechanism for collateral sensitivity to steroid hormones. The steroids would first sensitize the cells by somehow impairing the plasma membrane, a process requiring cholesterol. As a result, a hypersensitivity to ATPase activation and the following ETC mediated

ROS. These findings are important as they identify a mechanism that could be exploited to specifically target P-gp expressing MDR tumor cells.

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General Conclusion

Chemotherapy has been the main mode of effective treatment from a long time to combat against a wide variety of diseases with different sources (i.e. cancer, parasite, bacteria, viruses). Pharmaceutical research is in its constant progression, identifying new molecules with more specific mode of action. Nonetheless, chemotherapy remains an aggressive manner of treating a condition and side toxicity is often limiting the dosage require for complete efficiency of the treatment. Therefore, we see the appearance of drug resistance which could also be intrinsic. Various factors have been found to be implicated in drug resistance but it is really with the discovery of ABC transporter that the hope of solving this problem raised. Specifically, the implication of ABC transporter in clinical cancer drug resistance complicated to demonstrate. However, it is now accepted that at least three of them (ABCB1, ABCC1 and ABCG2) would be implicated [1]. In addition, P-gp along with BCRP are found in tumour stem cells that, in the new accepted way of viewing cancer, are believe to be the tumour initiator cells with self-renew property [2]. These cells are the most important one to target and because of their mentioned above properties, are more than likely to elicit intrinsic MDR and could be the major reason for relapse. For these reason, there is an urge to find a manner of targeting ABC transporters expressing cells. The goal is to understand a mechanism of action that could be exploited. Many efforts have been put to develop specific inhibitors of ABC transporters. Several inhibitors were produced but unfortunately they had limited success to improve patient survival. Alternative approach needed therefore to be explored. One of these approaches relies in the paradoxal collateral sensitivity elicited by some ABC expressing MDR cells. Indeed, some MRP1 or P-gp expressing cells show increase sensitivity to various compounds. Understanding the basic mechanism

of these phenomenons could pave the way for development of highly active compounds that would specifically targets ABC expressing MDR tumour cells.

The study presented in Chapter 2 described the molecular mechanism of collateral sensitivity of MRP1 expressing cells to GSH modulating agents. For demonstration we used the human SCLC selected for resistance to doxorubicin H69AR. Even before MRP1 was identified as the factor causing the resistance, these cells were shown to be collateraly sensitive to BSO [3]. BSO is a GSH synthesis inhibitor and it took a while to figure out a plausible hypothesis that would make sense. After MRP1 was discover, it is only with the finding that GSH was a substrate for MRP1 that coherent hypothesis took form [4]. The mechanism that could be drawn from chapter 2 is in accordance. We find that MRP1 expressing cells have a deficit in GSH due to the presence of MRP1. This deficit leads to hypersensitivity to the GSH synthesis inhibitor BSO. Also, when MRP1 mediated GSH extrusion is stimulated by Vrp or Api, the levels of GSH goes below a threshold where toxicity is observed. Being important for the maintenance of redox status, GSH depletion leads to the accumulation of ROS leading to apoptosis.

These results are important since they describe a mechanism with possibly clinically usable compounds. Indeed, BSO and Api are tolerated to a certain extend in human and mice with sera concentrations comparable to what was used here [5, 6]. Vrp is still sometimes clinically used for cardiac problems, although the concentrations used here would probably lead to toxicity. Nonetheless, additional results using drug combinations (not shown here) showed strong synergy between BSO and Vrp or BSO and Api. This synergy was specific for MRP1 expressing cells and would allow significant diminution of active concentration and therefore inducing less toxicity. At this point it would be interesting to test alone or in combination these compounds in mice with tumour xenograft expressing MRP1. Also, an oriented screening of compounds looking for the strongest collateral sensitivity would be of interest to perform. These compounds could be chosen according to their ability to

stimulate MRP1 GSH extrusion, inhibite GSH synthesis and inhibite ROS involved enzymes (GPX, Catalase and SOD).

Chapter 3 and 4 describe the elucidation of collateral sensitivity mechanism of P-gp expressing cells to Vrp and two steroid hormones Pro and DOC. Collateral sensitivity in CHO cell lines expressing P-gp was already observed in 1976 [7]. The colchicine resistant hamster cell line $CH^{R}C5$ was selected a year before. The compounds found at the time to induce collateral sensitivity varied (local anaesthetics, steroid hormones and Triton X). Later, many studies showed collateral sensitivity to the calcium channel blocker Vrp [8-12]. Among these, only one study (in another species than the hamster), they used the mouse cell line NIH3T3 [9]. It was shown that P-gp level of expression and MDR correlated with levels of collaterals sensitivity [10]. Also, partial elucidation of the mechanism pointed out the stimulation of P-gp ATPase as the mechanism for collateral sensitivity [12]. Nonetheless, a direct demonstration implicating P-gp in the mechanism was still missing. In addition other questions required explaination. Why is collateral sensitivity to Vrp found exclusively in some cell lines? Is there a general mechanism leading to collateral sensitivity with P-gp ATPase stimulator?

The findings presented in chapter 3 detail the mechanism of collateral sensitivity induced by Vrp. For the first time using siRNA experiments, P-gp was specifically identified as a prerequisite for this phenomenon. As we previously suggested we now confirm that Vrp stimulation of P-gp ATPase activity triggers higher demand of the ETC leading to ROS induced toxicity [12]. Data now quantified show that $CH^{R}C5$ cells elicit very high expression of sorcin protein while the parental AuxB1 but also the human Mcf7 and Mcf7/Adr do not show this. This protein sequestering intracellular Ca^{2+} leads to a significant increase of the latter level [8]. The abundant presence of sorcin could be the missing link explaining why only certain cells show collateral sensitivity. The latter hypothesis is reinforced by the fact that Vrp was shown to increase accumulation of Ca^{2+} [8]. The mechanism for this is not known but it is possible that it would do so through membrane permeation [13]. An

accumulation of calcium in cells that already contain high Ca^{2+} add to the ROS produced after the P-gp ATPase stimulation could easily lead to synergy and cell death. It is know that Ca^{2+} potentiates ROS mediated apoptosis.

In chapter 4 the P-gp ATPase stimulator Pro and DOC were studied for their ability to induce collateral sensitivity. As seen with Vrp and in accordance with previous results, they were bothshown to both induce collateral sensitivity in $CH^{R}C5$ cells [12]. Also, as seen with Vrp, the mechanism involves ETC mediated induced ROS and requires the presence of active P-gp. The combinations of Vrp and either Pro or DOC do not lead to additive toxicity effects. Interestingly, it seems that membrane cholesterol is essential for the collateral sensitivity. That, point out a first difference compared to the Vrp mechanism since we also showed that Vrp toxicity is not dependant on cholesterol. The cells (both the P-gp expressing and non-expressing cells) have intrinsic sensitivity to Pro and DOC. The P-gp ATPase activation leading to ETC mediated ROS potentiates the normal toxicity. As oppose to sorcin, which is express in few cells, cholesterol is present in all mammalian cells. Therefore, in accordance with the above proposed mechanism, the human cell line expressing P-gp, Mcf7/Adr, was also showing P-gp specific collateral sensitivity.

Together, the results showed in chapter 3 and 4 suggest a common trend, where the P-gp ATPase must be activated so that the energy demand over stimulate the ETC and ROS levels. Although, this is only one of the two minimum requirements. The ROS would indeed potentiate another aspect that is already present in some cells (Vrp/sorcin mechanism) or presumably most cells (Pro or DOC/cholesterol mechanism). It would be interesting to try other P-gp ATPase stimulators and see if other possible mechanisms of collateral sensitivity exist. These findings are of great interest because of the P-gp importance in MDR. They could be useful in designing specific therapies targeting P-gp expressing cells. However, some aspects of their mechanisms are still not well understood.

For the Vrp mechanism, it would be important to confirm the importance of sorcin in the Vrp one. To do so, one could simply target the expression of the gene encoding using siRNA and see the modulation of collateral sensitivity. Conversely, it would be interesting to see if sorcin transfection in other P-gp expressing cell line (including humans) would induce collateral sensitivity. Finally, it would be of interest to see the effect of other types of intracellular calcium modulation.

How cells are killed even without P-gp by Pro and DOC and how cholesterol is involved remains unclear. Cholesterol induces membrane rigidity and is implicated in raft formation [14]. Pro and DOC are steroids that are hydrophobic thus, it is possible that the mechanism relies on the physical properties of cell membranes. Therefore, it would be interesting to look at the influence of Pro or DOC on raft formation and disruption. Finally, it would be important to discriminate between raft and cholesterol in terms of their requirement for the collateral sensitivity. To do so one could use a raft disrupter which, contrary to the cyclodextrin used here, would not do so by removing cholesterol from the membrane.

Together, the results presented in this thesis elucidate three mechanisms of collateral sensitivity. With strategic use of this new knowledge I believe that they could lead to strong and specific ways of treating MRP1 and P-gp mediated MDR cancer. Knowing the mechanisms of action, correct screening of compounds could be done to find some that induce even stronger collateral sensitivity. I do believe that tumour stem cells could be targeted with this approach so we could eventually, once the most effective compound is found, use this approach as a first line chemotherapeutic.

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Appendix