Universidade de Aveiro Departamento de Biologia
Ano 2012

Susana Raquel Costa Barros

REDUÇÃO DO CITOCROMO C E RESISTÊNCIA AO METOTREXATO EM CANCRO DA MAMA

CYTOCHROME C REDUCTION AND METHOTREXATE RESISTANCE IN BREAST CANCER

Universidade de Aveiro Departamento de Biologia
Ano 2012

Susana Raquel Costa Barros

REDUÇÃO DO CITOCROMO C E RESISTÊNCIA AO METOTREXATO EM CANCRO DA MAMA

CYTOCHROME C REDUCTION AND METHOTREXATE RESISTANCE IN BREAST CANCER

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Carlos Julian Ciudad Gomez, Professor Catedrático do Departamento de Bioquímica e Biologia Molecular da Faculdade de Farmácia da Universidade de Barcelona e da Professora Doutora Maria da Conceição Lopes Vieira dos Santos, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro

The work was supported by grant SAF2011- 23582 from “Plan Nacional de Investigación Científica” (Spain)

I dedicate this work to my sister, Irina, to my brother in law, Danilo, to my uncle, Zé, and to my “scientific mother” Núria for all your efforts, in different ways, to make this project run.

o júri

Professor Doutor João António de Almeida Serôdio (Presidente)
Professor Auxiliar, Departamento de Biologia, Universidade de Aveiro

Doutor José Miguel Pimenta Ferreira de Oliveira (Arguente Principal)
Investigador Pós-Doutoramento, CESAM - Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro

Professor Doutor Carlos Julián Ciudad Gómez (Orientador)
Professor Catedrático, Departamento de Bioquímica e Biologia Molecular, Faculdade de Farmácia, Universidade de Barcelona

Professora Doutora Maria da Conceição Lopes Vieira dos Santos (Co-orientador)
Professora Associada com Agregação, Departamento de Biologia, Universidade de Aveiro

agradecimentos

I would like to express my deepest gratitude first and foremost to Prof. Dr. Maria da Conceição Santos for supporting my decision to go to Barcelona and joining this project. I'm also grateful for her friendship during these 2 years of Master.

I would also like to thank Prof. Dr. Carlos J. Ciudad and Dr. Veronique Noe for promptly accepting my application to join the CClab and this project and for everything they taught me: the scientific advices, knowledge and effort to make me a better scientist. I am especially very grateful to Carlos for providing me with enthusiastic scientific discussions and for his obvious care and friendship along the 6 months I spent in Barcelona. I consider myself very lucky to have had the opportunity of joining this project and meeting this great team at CCLab.

I am deeply indebted to Núria Mencía, my "scientific mother", for her patience, for sharing her knowledge and experience, even in the simplest things, and for her effort to make this project work! It was a great privilege to be "supervised" by someone like Núria, who you would love suddenly and never forget and who will remain my role model as a scientist!

I would also like to thank Laura, Carlota, Xenia and Anna Solé for all the good times we shared in the lab even when I was bad mooded and obviously for all the help and support. They are genuinely nice and supportive and I'm very glad to have met them and worked with them. Also, I would like to thank Gaspar, Anna Camps and Iris.

I wish to thank André, Ricardo, Xavi, Raquel, Juanito, Georgina and Betta, for all the good memories in Barcelona, their friendship and for teaching my basic knowledge of Spanish!

Since this project was run between Barcelona and Aveiro, I also wish to thank my colleagues from the LabCyt and my master courses' colleagues for their support and good moments.

I would also like to thank my friends Linda, Natacha, Nádia, Claudia, Joana Valdez, Maria and Joana Oliveira for their friendship and all the smiles they gave me in person or by phone/internet, especially during the last phase of this work the hardest one!

Last but not least, my deepest gratitude to my sister, Irina, Danilo and to my uncle Zé for making possible the dream of living and working in the great city of Barcelona for 6 months. Without you, this would have never been possible!

Thanks to my family for the enthusiasm, patience and care during this period of my life.

palavras-chave

citocromo c; estado redox; resistência ao metotrexato; apoptose; GSTs; GSH; cancro da mama

resumo

O metotrexato é um agente quimioterápico usado para tratar uma variedade de cancros. No entanto, a ocorrência de resistência limita a sua eficácia. A relação entre o estado redox do citocromo c, apoptose e o desenvolvimento de resistência ao metotrexato foi avaliada em células humanas de cancro da mama. O citocromo c no seu estado reduzido é menos capaz de induzir a cascata apoptótica. Aqui nós mostramos que células incubadas com agentes redutores de citocromo c, tais como tetrametilfenildiamina, ascorbato ou glutationa reduzida, demonstraram menos mortalidade e apoptose na presença do metotrexato. Por outro lado, a inibição de glutationa aumentou a acção apoptótica do metotrexato, demonstrando um envolvimento do estado redox do citocromo c na apoptose induzida por metotrexato. Usando ferramentas de genómica funcional detectámos que GSTM1 e GSTM4 foram sobreexpressos em células MCF7 resistentes ao metotrexato. A inibição destas isoformas causou um aumento na citotoxicidade em células sensíveis. Estes resultados sugerem que estas GSTs específicas juntamente com a glutationa reduzida endógena poderão ajudar a manter o citocromo c num estado mais reduzido o que por sua vez poderá causar uma diminuição na apoptose e contribuindo assim para a resistência ao metotrexato em células tumorais humanas.

keywords cytochrome c; redox state; methotrexate resistance; apoptosis; GSTs; GSH; breast cancer

abstract Methotrexate is a chemotherapeutic agent used to treat a variety of cancers. However, the occurrence of resistance limits its effectiveness. The relationship among redox-state of cytochrome c, apoptosis and development of resistance to methotrexate was assessed in human breast cancer cells. Cytochrome c in its reduced state is less capable of triggering the apoptotic cascade. Here we show that cells incubated with cytochrome c-reducer agents, such as tetramethylphenylenediamine, ascorbate or reduced glutathione, showed less mortality and apoptosis in the presence of methotrexate. Conversely, depletion of glutathione increased the apoptotic action of methotrexate, demonstrating an involvement of cytochrome c redox state in methotrexate-induced apoptosis. Using functional genomics we detected that GSTM1 and GSTM4 were overexpressed in methotrexate-resistant MCF7 breast cancer cells. Inhibition of these isoforms caused an increase in methotrexate cytotoxicity in sensitive cells. These results suggest that these specific GSTs together with endogenous reduced glutathione would help to maintain a more reduced state of cytochrome c which in turn would cause a decrease in apoptosis thus contributing to methotrexate resistance in human cancer cells.

Index

List of Figures

List of Tables

Acronym and abbreviations list

ALDH	Aldehyde dehydrogenase
AKR	Aldo-keto reductase
ASK	Apoptosis signal-regulating kinase
CAV1	Caveolin-1
DKK1	Dikkofp homolog 1
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
dTMP	Thymidine monophosphate
dUMP	Deoxyuridine monophosphate
EEF1A1	Eukaryotic translation elongation factor 1A1
ENO2	Enolase-2
ETC	Electron transport chain
FPGS	Folil polyglutamate synthase
GPx	Glutathione peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IAP	Inhibitor of apoptosis
IMM	Inner mitochondrial membrane
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
miRNA	microRNA
MDR	Multi-drug resistance
MRP	Multi-drug resistance protein
mtGPx	Mitochondrial glutathione peroxidase
MTX	Methotrexate

PBS	Phosphate Buffered Saline
PI	Propidium iodide
PKCα	Protein kinase c α
PPRH	Polypurine reverse-hoogsteen hairpin
PTP	Permeability transition pore
RFC	Reduced folate carrier
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SDS	Sodium Dodecyl Sulfate
SN	Supernatant
STP	Staurosporine
THF	Tetrahydrofolate
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
TS	Thymidilate synthase
UDPGA	Uridine diphosphoglucoronic acid
UGT	UDP-glucoronosyl transferase
VERA	Veratridine

Chapter I - General Introduction

1. Methotrexate and cancer treatment

1.1. Mechanism of action: cell cycle S phase arrest and apoptosis

Methotrexate (MTX) is an antifolate chemotherapeutic agent widely used, alone or in combination with other agents for the treatment of a range of cancers, such as breast cancer, osteosarcoma, head and neck cancer, lymphoma and acute lymphoblastic leukemia (Jolivet et al. 1983).

This type of drug and its polyglutamates inhibit competitively and reversely the dihydrofolate reductase (DHFR) affecting the synthesis *de novo* of purines and pirimidines, thus the treatment with MTX results in the DNA synthesis inhibition, causing p53 mediated cell cycle arrest in S phase and death cell generally by triggering apoptosis in several cell lines (Kaufmann 1989; Barry et al. 1990; da Silva et al. 1996; el Alaoui et al. 1997; Hattangadi et al. 2004). The absence of folates and nucleotides precursors causes changes in the DNA synthesis, once the carbon atoms exchange reactions are blocked. In this case, following the DNA damage caused by this type of anticancer drug, apoptosis may occur as a major event, where apoptotic regulating genes will influence the lethal effect of this drug or may be a secondary response to the induced drug cytotoxic effect (Brown and Wouters 1999; Tannock and Lee 2001). Tumor cells which proliferate abnormally are particularly susceptible to the lethal effects of the MTX.

DHFR catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) which is necessary, as well as its derivatives, as cofactor in different biochemical mechanisms involved in carbonyl groups transference, such as aminoacids (e.g. methionine) and the purines and pyrimidines synthesis as well. Thus, DHFR is a very important enzyme in the DNA replication and cell division, by keeping the intracellular reduced folate levels. Also thymidylate synthase (TS) has an important role in DNA synthesis, since it catalyzes thymidine monophosphate formation (dTMP) from deoxyuridine monophosphate (dUMP), precursor of pyrimidines.

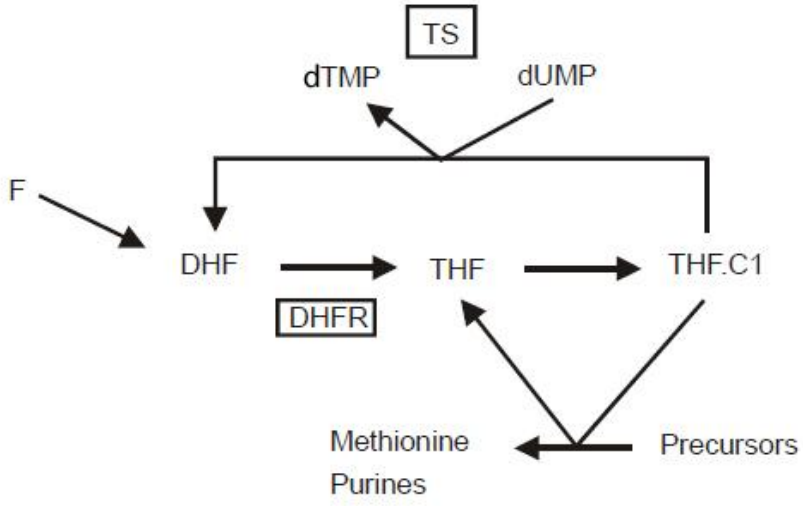
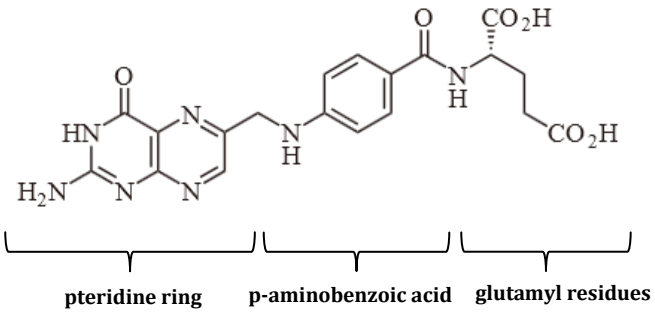


Figure 1– Schematic representation of folates (F) metabolism. Reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) catalyzed by dihydrofolate reductase (DHFR). THF.C1 represents THF active derivatives that participate in specific folate-dependent reactions. (Adapted and modified from unknown source)

The natural folates are formed by three structural components: a ptheridin ring, an acid p-aminobenzoic and a glutamate (Figure 2a). The antifolates also share this structure however with slight variations (Figure 2b).

a



b

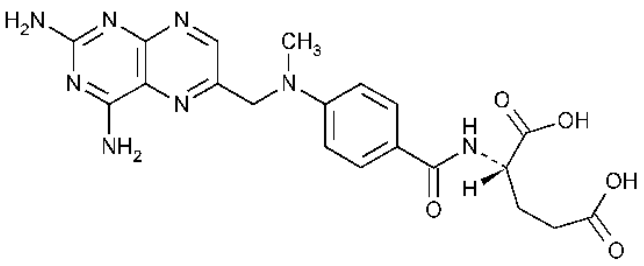


Figura 2– Chemical structure of natural folates (a) and methotrexate (antifolate) (b).

1.1. Mechanisms of MTX resistance

The occurrence of resistance to MTX upon treatment compromises its effectiveness, limiting its use in chemotherapy (Hryniuk and Bertino 1969) and its occurrence can be either by a single mechanism or by combination of several of them (Sharma et al. 1991), thus drug resistance is a complex process in which several pathways can be involved. Several resistance mechanisms *in vitro* have been described using mammalian cells as model. Following, a brief description of each mechanism:

1.2.1. Gene amplification of the *DHFR* locus

One explanation for the amplification of the *DHFR* gene copy number could be due to unequal sister chromatid exchanges generating stable chromossomic regions. It has also been described that the generation of extrachromossomic copies of the *DHFR* gene, normally lost in daughter cells because of the absence of the centromere, is associated to the amplification process (Schimke et al., 1981). Other studies suggested a direct relationship between the amplication of the *DHFR* gene and mutated *p53* gene (Livingstone et al. 1992).

1.2.2. Deficiency in MTX transport

MTX transport into the cell occurs mainly through a specific folate/antifolate transporter named reduced folate carrier (RFC). RFC is a membrane glycoprotein encoded by *SLC19A1* gene. Underexpression of human *SLC19A1* gene seems to be the central mechanism associated to the deficiency in MTX transport (Gorlick et al. 1997; Belkov et al. 1999; Guo et al. 1999; Ferreri et al. 2004) probably caused by the loss or deletion of locus *SLC19A1* (Ding et al. 2001; Zhao et al. 2004). Another common mechanism associated to resistance development is the occurrence of *SLC19A1* gene mutations, changing the transporter function, thus causing a decrease in MTX transport and originating the drug resistance (Roy et al. 1998; Rothem et al. 2002).

1.2.3. Reduction in the MTX polyglutamation

Polyglutamation is a biochemical process with an important role in the MTX pharmacology (Rosowsky et al. 1982). Like for the natural folates, folil polyglutamate synthase (FPGS) has high affinity to MTX adding sequentially five groups of glutamate to its molecule. Once it has more than three glutamate groups, MTX is no longer substrate for the RFC transport system, causing the accumulation of polyglutamated MTX inside the cell (Cowan and Jolivet 1984). This polyglutamated form of MTX is further a stronger inhibitor of the synthesis of purines and pyrimidines (Kimura et al. 2004) by DHFR and other THF-dependent enzymes inhibition (Cheok and Evans 2006). This inhibition can still remain even after the chemotherapeutic treatment, increasing the cytotoxic activity of the MTX (Cowan and Jolivet 1984).

The occurrence of less polyglutamation had been described in resistant cells (McCloskey et al. 1991; Mauritz et al. 2002; Liani et al. 2003). It is already described that several tumor cell lines resistant to antifolates show a suppression in the FPGS activity, probably due to post-translational alterations or gene mutations (Roy et al. 1997), thus the decrease in the accumulation of polyglutamated MTX can be associated to a low activity of FPGS.

1.2.4. Increase of the multi-drug resistance phenotype

The development of drug resistance in chemotherapy has been related to several mechanisms and it may occur as a multifactorial event where several mechanisms are simultaneously activated. The appearance of the multi-drug resistance (MDR) phenotype is a great concern regarding the chemotherapeutic treatment in several types of cancer (Sharom 1997). This phenotype can be inherited, an innate property of tumor cells, or acquired upon drug treatment (Sharom 1997). Its development can be associated to classic mechanisms, directly involved in DNA replication or detoxification processes: 1) the classic MDR phenotype can result from the overexpression of the p-glycoprotein, encoded by *mdr1* gene, and from other multidrug resistance proteins (MRP) involved in the decrease of intracellular drug concentration through its ATP-dependent efflux. The

p-glycoprotein is an ATP-dependent translocase known for its ability to carry chemotherapeutic drugs, such as MTX (Sharom 1997); 2) the development of non-classic MDR phenotype is normally associated to alterations in the levels or activity of topoisomerase II and overexpression of detoxification enzymes phase II such as glutathione S-transferases (GSTs), UDP-glucuronosyl transferases (UGTs), aldehyde dehydrogenases (ALDHs) and aldo-keto reductases (AKRs) (Ax, 2000; Inoue, 1993). However, this type of MDR phenotype could be masked by the activation of the drug efflux via MDR or MDP.

Hereupon, it has been supposed that the increase of the MDR phenotype could be associated to alterations in the functional p-glycoprotein caused by the exposure to high doses of MTX (Assaraf et al. 1989). It is also described that the overexpression of different members of MRP family provides resistance to antifolates, although it is not described MRP overexpression in antifolate selected cell lines (Assaraf 2007).

1.2.5. Mutations of the *DHFR* gene

Mutations in *DHFR* gene in resistant cell lines decrease the affinity of DHFR protein to MTX compromising its inhibition by this drug, thus favoring the occurrence of the resistance (Srimatkandada et al. 1989).

1.2.6. Altered genes

Besides *DHFR* gene, other genes have been identified also important in the development of the MTX resistant phenotype, such as *AKR1C1, UGT1A6*, *DKK1*, E-cadherin, caveolin 1, enolase-2, *PRKCA*, *EEF1A1* and *S100A4*.

1.2.6.1. Overexpression of *AKR1C1*

Aldo-keto reductase (AKR) superfamily proteins are monomeric cytoplasmic proteins and have been suggested to be involved in detoxification processes (Maser 1995; Burczynski et al. 1999; O'Connor et al. 1999; Penning

2005) by catalyzing the NAD(P)H-dependent oxido-reduction of a variety of substrates (Jez et al. 1997).

Overexpression of *AKR1C1* has been related to drug resistance in a range of cancers. Some anticancer drugs share similar chemical structure with some compounds metabolized by AKR1C1, suggesting that these drugs may be subject to this enzyme activity (Hsu et al. 2001). Several studies using different anti-cancer drugs are in concordance with this, altogether suggesting an association between AKR1C1 and drug detoxification (Ciaccio et al. 1993; Shen et al. 1997; Ax et al. 2000; Deng et al. 2004; Chen et al. 2005; Hung et al. 2006). Also MTX-resistant colon cancer cells showed an overexpression of *AKR1C1* (Selga et al. 2008). In this study, *AKR1C1* overexpression seems to represent a mechanism, parallel to *DHFR* amplification, to the development of MTX resistance, once *AKR1C1* up-regulation counteracted MTX-induced S phase arrest of cell cycle and apoptosis caused by the drug.

1.2.6.2. Overexpression of *S100A4*

S100A4 belongs to the S100 calcium binding protein family and as many members of this family, S100A4 is a symmetric homodimer characterized by the presence of two calcium binding sites of EF-hand type (helix-loop-helix) (Dukhanina et al. 1997) that allow S100 proteins to respond to calcium stimulus induced by cell signaling. It has been involved in the regulation of a wide variety of cellular processes, such as protein phosphorylation, dynamics of cytoskeleton constituents or Ca^{2+} homeostasis (Donato 2001; Donato 2003). S100A4 has been described to have a function in cell cycle progression, in cell motility and as modulator of intracellular adhesion and of the invasive properties of cells (Sherbet and Lakshmi 1998; Donato 2001). Its overexpression has been associated to tumor malignancy (Parker et al. 1994), metastasis (Lloyd et al. 1998), angiogenesis (Ambartsumian et al. 2001) and drug resistance as well (Mahon et al. 2007).

Mencia et al. (2010) demonstrated that *S100A1* is overexpressed in several MTX-resistant colon cancer, breast cancer, pancreatic cancer, leukemia and osteosarcoma cell lines. This study also showed that cellular knockdown of *S100A4* in parental cells leads to chemosensitization toward MTX and overexpression

desensitizes the cells toward this drug, suggesting a role for S100A1 in MTX-resistance in colon cancer cells.

1.2.6.3. Overexpression of caveolin-1, enolase-2 and *PRKCA* and decrease of E-Cadherin-1 expression

Caveolin-1 (CAV1) is the main component of the caveolae membrane system and is known to control cell proliferation and viability by suppressing survivin, a member of IAP (inhibitor of apoptosis) family via a transcriptional mechanism involving β-catenin-Tcf/Lef-1 pathway (Torres et al. 2007). This protein function has been found to be involved in tumor progression and invasiveness (Thompson 1998; Yang et al. 1998; Ho et al. 2002) and also associated to multidrug resistance (Lavie, 1998). Studies have demonstrated that *CAV1* is overexpressed in MTX-resistant colon cancer cell lines, showing a role for CAV1 in MTX resistance (Bender et al. 2000; Selga et al. 2008).

Enolase-2 (ENO2) is glycolysis-related and is induced by hypoxia, an intrinsic tumorigenic property. It has been described that ENO2 plays an important role in tumorogenesis of colorectal cancers (Yeh et al. 2008) and it is upregulated in a variety of cancers (Fujiwara et al. 2002; Karnak et al. 2005; Kitakata et al. 2007). Selga et al. (2008) showed a role for ENO2 in the MTX resistance when noticed an increase in sensitivity to MTX after transfected with a siENO2.

Protein kinase c α (PKCα) has also been associated with multidrug resistance (Yu et al. 1991). The α-isoenzyme PKC phosphorylates different proteins promoting a wide variety of cellular responses including proliferation, differentiation, membrane transport, gene expression and tumor promotion (Martelli et al. 1999; Wang et al. 1999). Using chemical inhibitors of PKC activity, Noé et al. (1995) have been proposed PKC as resistance modulators in MTX chemotherapy. Moreover, reducing *PKCα* mRNA levels diminishes the MDR phenotype in tumor cells (Ahmad and Glazer 1993) and sensitizes cells to anticancer drugs, both in vitro (Wang and Liu 1998; Isonishi et al. 2000; Lahn et al. 2004) and in vivo (Geiger et al. 1998). Selga et al. (2008) also demonstrated an

increase of sensitization toward MTX with a decrease in *PKCα* mRNA levels, revealing a role for PKCα in the MTX resistance.

A direct interaction has been described between PKCα and CAV1 (Oka, Yamamoto et al. 1997). *PKCα* overexpression may represent an important cellular event in tumor progression, once in MCF-7 breast cancer cells transfected with *PKCα*, the expression of E-cadherin and β-catenin decrease, resulting in a loss of cell-cell adhesion and thus in a more aggressive tumor phenotype (Lahn et al. 2004).

The loss of E-cadherin is frequently associated with tumor progression (Behrens et al. 1989; Perl et al. 1998) and is considered a central event that stimulates metastasis and invasiveness of the tumors (Frixen et al. 1991; Cavallaro and Christofori 2004). MTX-resistant colon cancer cells showed an underexpression of E-cadherin (Selga et al. 2008).

1.2.6.4. Overexpression of *DKK1* and *EEF1A1*

Dikkofp homolog 1 (DKK1) is a protein involved in embryonic development (Forget et al. 2007) and has also been described as an inhibitor of Wnt signaling (Rothbacher and Lemaire 2002). A role of its overexpression has been suggested in cancer (Monaghan et al. 1999; Gregory et al. 2003; Koch et al. 2005; Forget et al. 2007; Voorzanger-Rousselot et al. 2007) however the precise mechanism is poorly understood. In MTX-resistant colon cancer cells the role of DKK1 is still unclear, but it seems to be related to the resistant phenotype (Selga et al. 2009). Also Katula et al. (2007) demonstrated that MTX inhibited *DKK1* transcription, corroborating with the hypothesis that *DKK1* overexpression could constitute a mechanism to overcome the MTX inhibitory effect over the transcription.

Eukaryotic translation elongation factor 1A1 (EEF1A1) is an elongation protein factor that recruits aminoacetylated tRNAs to the A site of the ribosome (Thornton et al. 2003) and also its overexpression has been found in a variety of cancers (Alon et al. 1999; Thornton et al. 2003). It is already described that

EEF1A1 expression is involved in increased cell proliferation (Hassell and Engelhardt 1976; Grassi et al. 2007), oncogenic transformation (Tatsuka et al. 1992), delayed senescence (Shepherd et al. 1989) and metastasis (Edmonds et al. 1996). Furthermore, its overexpression has also been associated with MTX resistance (Beyer-Sehlmeyer et al. 1999) and to drug resistance toward other chemotherapeutic agents as well (Bertram et al. 1998; Johnsson et al. 2000), probably due to its inhibitory activity over apoptosis (Talapatra et al. 2002). Selga et al. (2009) verified that the knockdown of *EEF1A1* by small interfering RNA (siRNA) technology in pancreatic cancer cell line sensitized the cell to MTX, revealing a role for EEF1A1 in MTX resistance.

1.2.6.5. Increase in UGT1A family expression

UDP-glucoronyltransferases are a family of enzymes involved in phase II metabolism, responsible for the glucoronidation of many lipophilic endogenous substrates such as bilirubin, estrogens, and xenobiotics. The addition of a glycosyl group from uridine diphosphoglucoronic acid (UDPGA) returns hydrophobic compounds more soluble for their elimination via bile and urine.

Selga et al. (2009) showed that there is an overexpression in UGT1A family in human breast cancer cells resistant to MTX. UGT1A6, among different members of UGT1A family, could be the main responsible for the rise of *UGT1A* expression (Selga et al. 2009). UGT1A6 is the main UGT that mediates glucoronidation in human (Krishnaswamy et al. 2005). As MTX shares a phenolic structure common to other UGT1A substrates, UGT1A family could somehow contribute to MTX metabolism, thus contributing to resistance. It is also described that *UGT1A* overexpression in MTX resistant cell lines is associated with an increase in *UGT1A* transcription caused by MTX action as an inducer of increased mRNA levels and not due to gene amplification. This induction could occur through MTX action on some transcription factors involved in *UGT1A* induction, such as ARNT and AhR/ARNT (Selga et al. 2009).

1.2.7. Altered miRNA expression: underexpression of miR-224

MicroRNAs (miRNAS) are a new class of small non-coding RNAs involved in RNA silencing that play a role in many biological processes (Carrington and Ambros 2003; Bartel 2004). They are involved in the development of many diseases, including cancer (Calin et al. 2002; Metzler et al. 2004; Cimmino et al. 2005; Hayashita et al. 2005; Iorio et al. 2005; Murakami et al. 2006; Voorhoeve et al. 2006). Extensive studies show that miRNAs play a role in cancer pathogenesis and in the development of drug resistance as well (Ma et al. 2010).

Mencia et al. (2011) showed that miR-224 is greatly underexpressed in MTX-resistant colon cancer cells. In this work, the underexpression of miR-224 seems to be responsible for the increase of mRNA levels of *SLC4A4*, *CDS2* and *HSPC159* genes in colon cancer MTX-resistant cells, thus contributing to less MTX cytotoxicity. This was confirmed when using Polypurine reverse-Hoogsteen hairpins (PPRHS) and siRNAs to knock down the miR-224 targets the cells became more sensitive to the MTX. SLC4A4 is a membrane transport protein responsible for transport of sodium and bicarbonate across the membrane in epithelial cells (Romero et al. 1997). As described above, alterations in membrane transport have shown to be a determining factor for the development of MTX resistance (Zhao and Goldman 2003). Differential expression of *SLC4A4* can induce changes in pH affecting the optimal activity of RFC and therefore decreasing the amount of MTX inside the cell, favoring the resistance phenotype (Mencia et al. 2011). *CDS2* encodes an enzyme that is responsible for the conversion of phospatidic acid to CDP-diaglycerol, thus regulating the amount of phosphatidylinositol available for signaling (Weeks et al. 1997). Breakdown products of phosphoinositides are ubiquitous second messengers involved in important cellular processes such as cell growth and protein kinase C activity. The role of PKCα has already been associated with resistance to MTX (Selga et al. 2008). Its inhibition caused chemosensitization to MTX, thus playing an important role in MTX resistance in colon cancer cells (Mencia et al. 2011). HSPC159 (galectin-related protein) is part of the galectin family but it lacks the capacity to bind β-galactosides (Cooper and Barondes 1999; Cooper 2002) and its biological function still remains unknown.

These results demonstrate that besides all the mechanisms described before, the underexpression of miR-224 in its targets expression *SLC4A4*, *CDS2* and *HSPC159* leads to insensitivity towards MTX, favoring the resistant phenotype.

2. Apoptosis in cancer and chemotherapy: p53 as an apoptotic mediator

Deregulated proliferation and inhibition of apoptosis are the main alterations during cancer development inherent to an accumulation of oncogenic mutations and obviously these two altered processes represent important targets for therapy (Evan and Vousden 2001). Deregulation of both phenomena is a complex process involving numerous coupled key mechanisms of oncogenic proliferative signals and suppression of apoptosis.

Originally apoptosis was described by Kerr et al. (1972) as a defined highly regulated form of programmed cell death characterized by progressive activation of specific pathways toward precise morphological and biochemical changes in the cell without involving an inflammatory response (Table 1).

These morphological changes lead to the packaging of the dead cells into apoptotic bodies. Apoptotic bodies are subsequently recognized and swallowed by phagocytic cells. As a gene-directed program, apoptosis is a crucial process to maintain the cell´s homeostasis under physiological conditions, since it seems to be induced when injury exceeds the repair ability, as well as during the normal tissue development and like any other metabolic or developmental program, it can be disrupted by mutation. In fact, its deregulation is associated either as a cause or consequence of several pathologies ranging from neurodegenerative disorders to malignancy.

Alterations in p53 pathway appear to be central in cancer development. p53 ability to eliminate damaged cells by apoptosis is vital to regulate the cell proliferation in multi-cellular organisms (Huang and Strasser 2000) and can be activated by external or internal stimuli. Due to damaged DNA, expression of oncogenes, hypoxia and nucleotide depletion (Giaccia and Kastan 1998), p53 is activated and its transcriptional activity can induce the expression of pro-apoptotic proteins and activate proteins involved in cell growth arrest.

Apoptotic cell death can occur by at least two distinct molecular signaling pathways based on the origin of apoptotic signal: extrinsic pathway activated by extracellular signals and intrinsic pathway that involves mitochondrial mediation and subsequent cytochrome c release, both activating the caspases, proteolytic enzymes responsible for dismantling the cells.

Most of chemotherapeutic DNA-damaging agents act primarily as apoptotic-inducers in the target cells. DNA-damaging agents apoptotic action is associated to activation of p53, which in turn stimulates the expression of Bcl-2 family members involved in the mitochondrial pathway (Fisher 1994; Kaufmann and Earnshaw 2000).

2.1. Apoptotic intrinsic pathway: cytochrome c release and MTX

MTX, as DNA-damaging agent, activates the p53 pathway triggering apoptosis by induction of some proteins involved in the mitochondrial or intrinsic pathway, such as Bax and Bid.

Currently, it is widely accepted that mitochondria play a key role in the regulation of apoptosis and has a main role in the apoptotic intrinsic pathway. Upon anticancer drugs treatment, mitochondria are induced to release cytochrome c. This process is dominated by the Bcl-2 protein family (Cory and Adams 2002; Kuwana et al. 2002). This family includes anti-apoptotic proteins, such as Bcl-X_L, and pro-apoptotic proteins, such as Bax and Bid. The later proteins are p53 targets. In response to induced-stress, Bax forms a homo-oligodimer and interacts with of permeability transition pore (PTP) components causing the opening of non-specific pores in outer mitochondrial membrane (OMM) and subsequent cytochrome c release (Narita et al. 1998). Mitochondrial membrane permeabilization therefore appears to be a key initiative step in the apoptotic process. Once released from the mitochondria into cytosol cytochrome c interacts with Apaf-1 recruiting and activating pro-caspase-9, forming the apoptosome (Zou et al. 1999).

Table 1 – Cellular changes during the apoptotic process
(Kerr et al. 1972)

Apoptosis
Morphological
Asymmetric membrane without loss of integrity
Aggregation of chromatin in the nuclear envelope
Condensation of cytoplasm and nuclei
Vesicle formation
Apoptotic bodies
Mitochondria become permeable
Biochemical
Active process
Signals transduction cascades activation: activation of caspase cascade
Non aleatory fragmentation of DNA
Physiological
Individual cells affected
Induced by physiological stimuli
Phagocytosis mostly by macrophages
With no inflammatory response

Activated caspase-9, in turn, cleaves and activates caspase-3 and -7 (Kim et al. 2005). These effector caspases are responsible for the activation of several key proteins leading to apoptotic characteristic biochemical and morphological changes (Robertson et al. 2000).

3. Regulation of apoptosis by cytochrome c redox-state

Cytochrome c release from the mitochondria into the cytosol is the key event in the apoptotic intrinsic pathway and even though it is irreversible, some evidences suggest that the execution phase of apoptosis is highly regulated after cytochrome c release (Twiddy et al. 2004; Martin et al. 2005).

Cytochrome c is a heme-protein traditionally localized in the mitochondrial inter-membrane and it is an essential component of the electron transport chain (ETC), being responsible for shuttling electrons between complexes III and IV. Cytochrome c exists in interconvertible reduced (heme Fe^{2+}) or oxidized (haem Fe^{3+}) forms. These two forms have different physical and biochemical properties (Martin et al. 2005). Reduced cytochrome c is less capable of binding to anions and binds less tightly to negatively charged membrane (Hancock et al. 2001) and this may influence the kinetics of activation of the apoptosome (Brown and Borutaite 2008).

Several studies where cytochrome c in its two different redox states was added to cell extracts demonstrated that oxidized cytochrome c induced apoptotic activity and reduced cytochrome c had no effect, measured by nuclear fragmentation or caspase-9 and -3 activities (Pan et al. 1999; Suto et al. 2005; Borutaite and Brown 2007).

Borutaite and Brown (2007) demonstrated that cytochrome c when added to cytosolic extracts, was partially reduced and when further reduced using agents such as tetramethylphenylenediamine (TMPD) or ascorbate also the caspase activation was inhibited, whereas when cytochrome c oxidase was added to oxidize cytochrome c, caspase activation was enhanced.

All these studies are in agreement with each other, showing that reduced cytochrome c is little or not able to induce caspase activation in cytosol, whereas oxidized cytochrome c induces caspase activation – Figure 3.

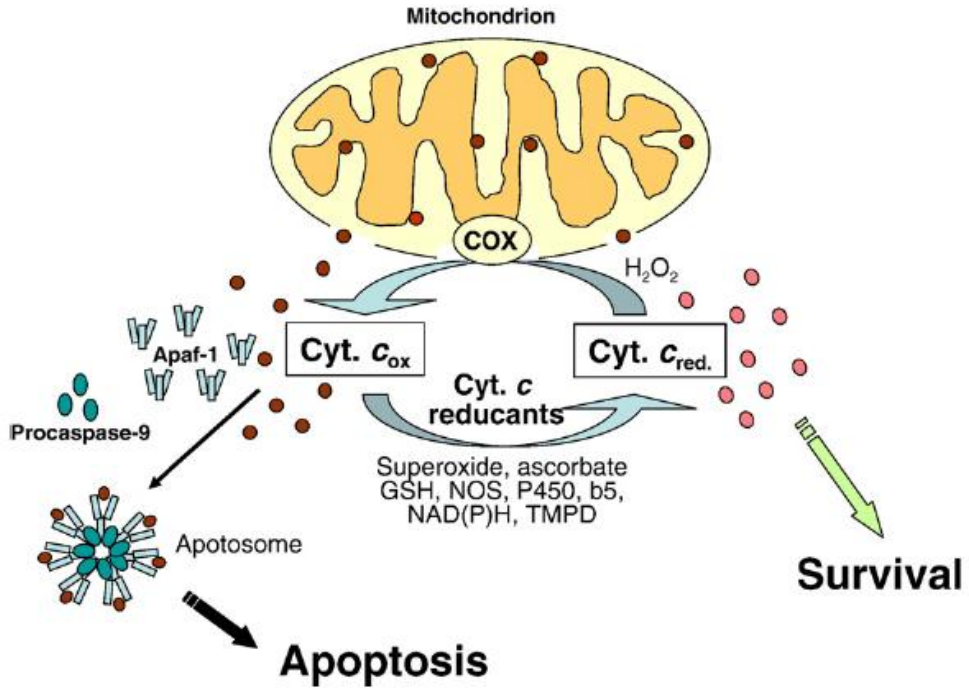


Figure 3 – Regulation of apoptosis by the redox state of cytosolic cytochrome c. Cytochrome c in its oxidized form (Cyt. C_{ox}) is released, binding to Apaf-1 forming the apoptosome which activates pro-caspase-9 leading to apoptosis. Cytochrome c can be reduced by several reductants, which cannot activate the apoptosome and therefore cannot promote apoptosis. (Adapted from Brown&Borutaite, 2008)

Also the different redox forms of cytochrome c might have different binding affinities for Apaf-1 and different abilities to activate Apaf-1, after binding, whereas the reduced form of cytochrome c would have lower affinity to Apaf-1. Activation of Apaf-1 could be blocked by reduced cytochrome c when it binds to ATP needed for Apaf-1 activation (Chandra et al. 2006), thus the apoptosome formation, inhibiting apoptosis.

However, Hampton (1998) and Kluck (1997) have demonstrated that cytochrome c redox state did not affect the caspase activation, thus the apoptosis by caspase; still they found that its activation was dependent on some structural features of cytochrome c.

Furthermore, it appears that the redox state of cytochrome c may also regulate apoptosis upstream of caspase activation. Redox state of cytochrome c is

strongly regulated by the cellular redox system. In mitochondria, cytochrome c is in its proper location, under physiological conditions, due to an interaction with cardiolipin, preventing its release to the cytosol (Ott et al. 2007). Since cardiolipin is sensitive to lipid peroxidation, when the ROS-scavenging activity of mitochondrial GSH and mitochondrial glutathione peroxidase (mtGPx) is compromised, excessive ROS can activate the peroxidase activity of cytochrome c, leading to cardiolipin peroxidation (Kagan et al. 2005). Oxidized cardiolipin loses its affinity to cytochrome c, favoring its release from the mitochondria. This could be one way in which the cytochrome redox state regulates apoptosis prior cytochrome c release, since the reduced form of cytochrome c is unlikely to be capable of oxidizing cardiolipin.

3.1. Cytosolic GSH and cytochrome c redox state

Glutathione (L-g-glutamy-L-cysteinylglycine - GSH) is the most abundant intracellular tripeptide thiol and it is well established that it is one of the most important components in the antioxidant defense system and a major detoxification agent in cells via catalysis by GST and glutathione peroxidases (GPx) (Clark et al. 1984), maintaining the optimal redox environment for normal activity of the cellular proteins. Under oxidative conditions, reduced GSH is oxidized to glutathione disulfide (GSSG) and then reverted to GSH by glutathione reductase (GR) activity (Ames 1989; Ames et al. 1993).

Beyond its antioxidant and detoxification activity, it has been also described the role of GSH in apoptosis in a variety of cell types (Pias et al. 2003; Okouchi et al. 2006; Circu and Aw 2008). Apoptosis can be induced by many factors, including the presence of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) or superoxide (O_2^-) that can arise from a variety of cellular sources, such as electron leakage in mitochondria. Generally, ROS-mediated apoptosis is associated with a decrease in GSH levels in the cell, including mitochondrial GSH and the loss of intracellular redox balance. ROS production associated to the decrease of mitochondrial GSH levels provokes the loss of mitochondrial membrane potential inducing cytochrome c release. It has also been described that the intracellular GSH decrease may induce the cytochrome c release, through redox regulation of PTP

opening and Bax translocation to mitochondria (Ghibelli et al. 1999; D'Alessio et al. 2005).

The released cytochrome c, is a key component for the triggering of the intrinsic pathway of apoptosis and, since it can exist in two redox forms, reduced or oxidized, it can also be influenced by the redox environment in the cytosol (Hancock et al. 2001). As stated before, once in the cytosol, cytochrome c needs to be in its oxidized state to induce apoptosis. Depletion of cytosolic GSH would induce the pro-apoptotic action of cytochrome c, since the cytosolic environment will be more oxidized and therefore the cytochrome c may be oxidized, triggering the apoptosis (Brown and Borutaite 2008). Under physiological conditions, the high GSH in cytosol will keep cytochrome c in a reduced state, whereas if the cell has been exposed to oxidizing conditions the cytochrome c becomes oxidized. Vaughn and Deshmukh (2008) have demonstrated that in healthy neurons and cancer cells, cytochrome c is kept in its reduced state, thus held inactive by increased GSH levels, generated by glucose metabolism by pentose phosphate pathway.

GSH also protects proteins from irreversible oxidation by forming mixed disulfide linkages with cysteines in proteins. This is called s-glutathionylation and it is important to regulate protein function mediated by GSTs (Fratelli et al. 2002; Dalle-Donne et al. 2005; Shelton et al. 2005). Although there is no evidence of s-glutathionylation of cytochrome c *in vivo*, it has been demonstrated a direct interaction between cytochrome c and GSH *in vitro* (Deng 2006).

4. Role of GSTs and GSH in anti-cancer drug-resistance

GST family is part of cellular Phase II detoxification system composed by enzymes that catalyse the conjugation of GSH to a variety of endogenous and exogenous electrophilic compounds, protecting cellular macromolecules from their attack (Hayes et al. 2004).

Human GSTs can be divided into three main super-families: cytosolic, mitochondrial and membrane-bound microsomal. Human cytosolic GSTs are extremely polymorphic and are distributed into seven classes initially based on Greek alphabet (newer nomenclature uses Latin script) alpha (A), Mu (M), Omega

(O), Pi (P), Sigma (S), Theta (T) and Zeta (Z) (Hayes and Pulford 1995; Armstrong 1997; Hayes and McLellan 1999; Hayes, Flanagan et al. 2004). This classification is essentially based on > 60% share sequence identity within a class, focused on the more highly conserved N-terminal domains containing catalytically active tyrosine, cysteine or serine residues.

The catalytic functions of GSTs in conjugating GSH with a variety of electrophilic substrates is well described (Boyland and Chasseaud 1969). Apart from its functions in detoxification, GSTs also play a role in death signaling regulation via mitogen-activated protein kinase (MAPK) pathway, inducing either extrinsic or intrinsic apoptotic pathway when the cell is under oxidative stress (Adler et al. 1999). The GSTs regulation lies in the interaction between c-Jun N-terminal kinases (JNK) and apoptosis signal-regulating kinase (ASK). This interaction is activated by GST-mediated negative regulation in response to cellular stress. ASK1 is a mitogen-activated protein kinase kinase kinase responsible for activation of JNK and p38 pathways leading to stress-induced apoptosis. Both *JNK* and *ASK1* are maintained in a low level in non-stressed cells due to the interaction with GST. GST overexpression blocked ASK1 oligomerization, repressing ASK1-dependent apoptotic cell death via JNK-pathway, since JNK is responsible for phosphorylation of transcription factors involved in apoptosis (Cho et al. 2001). This can possibly explain the resistant phenotype toward chemotherapy agents in some drug resistant cells, even when the drug is not a substrate for GST-mediated conjugation to GSH. A variety of anticancer agents induce apoptosis via JNK and p38 pathways.

Therefore, GSTs can have two different roles in the development of drug resistance: via direct detoxification as well as inhibiting MAPK-induced apoptosis, thus a large number of tumor types exhibit high levels of GSTs and GSH as well.

5. References

Adler, V., Z. Yin, et al. (1999). "Regulation of JNK signaling by GSTp." EMBO J **18**(5): 1321-1334.

Ahmad, S. and R. I. Glazer (1993). "Expression of the antisense cDNA for protein kinase C alpha attenuates resistance in doxorubicin-resistant MCF-7 breast carcinoma cells." Mol Pharmacol **43**(6): 858-862.

Alon, U., N. Barkai, et al. (1999). "Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays." Proc Natl Acad Sci U S A **96**(12): 6745-6750.

Ambartsumian, N., J. Klingelhofer, et al. (2001). "The metastasis-associated Mts1(S100A4) protein could act as an angiogenic factor." Oncogene **20**(34): 4685-4695.

Ames, B. N. (1989). "What are the major carcinogens in the etiology of human cancer? Environmental pollution, natural carcinogens, and the causes of human cancer: six errors." Important Adv Oncol: 237-247.

Ames, B. N., M. K. Shigenaga, et al. (1993). "Oxidants, antioxidants, and the degenerative diseases of aging." Proc Natl Acad Sci U S A **90**(17): 7915-7922.

Armstrong, R. N. (1997). "Structure, Catalytic Mechanism, and Evolution of the Glutathione Transferases." Chemical Research in Toxicology **10**(1): 2-18.

Assaraf, Y. G. (2007). "Molecular basis of antifolate resistance." Cancer Metastasis Rev **26**(1): 153-181.

Assaraf, Y. G., A. Molina, et al. (1989). "Cross-resistance to the lipid-soluble antifolate trimetrexate in human carcinoma cells with the multidrug-resistant phenotype." J Natl Cancer Inst **81**(4): 290-294.

Ax, W., M. Soldan, et al. (2000). "Development of daunorubicin resistance in tumour cells by induction of carbonyl reduction." Biochem Pharmacol **59**(3): 293-300.

Barry, M. A., C. A. Behnke, et al. (1990). "Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia." Biochem Pharmacol **40**(10): 2353-2362.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-297.

Behrens, J., M. M. Mareel, et al. (1989). "Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion." J Cell Biol **108**(6): 2435-2447.

Belkov, V. M., E. Y. Krynetski, et al. (1999). "Reduced folate carrier expression in acute lymphoblastic leukemia: a mechanism for ploidy but not lineage differences in methotrexate accumulation." Blood **93**(5): 1643-1650.

Bender, F. C., M. A. Reymond, et al. (2000). "Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity." Cancer Res **60**(20): 5870-5878.

Bertram, J., K. Palfner, et al. (1998). "Overexpression of ribosomal proteins L4 and L5 and the putative alternative elongation factor PTI-1 in the doxorubicin resistant human colon cancer cell line LoVoDxR." Eur J Cancer **34**(5): 731-736.

Beyer-Sehlmeyer, G., W. Hiddemann, et al. (1999). "Suppressive subtractive hybridisation reveals differential expression of serglycin, sorcin, bone marrow proteoglycan and prostate-tumour-inducing gene I (PTI-1) in drug-resistant and sensitive tumour cell lines of haematopoetic origin." Eur J Cancer **35**(12): 1735-1742.

Borutaite, V. and G. C. Brown (2007). "Mitochondrial Regulation of Caspase Activation by Cytochrome Oxidase and Tetramethylphenylenediamine via Cytosolic Cytochrome c Redox State." Journal of Biological Chemistry **282**(43): 31124-31130.

Boyland, E. and L. F. Chasseaud (1969). "The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis." Adv Enzymol Relat Areas Mol Biol **32**: 173-219.

Brown, G. C. and V. Borutaite (2008). "Regulation of apoptosis by the redox state of cytochrome c." Biochim Biophys Acta **1777**(7-8): 877-881.

Brown, J. M. and B. G. Wouters (1999). "Apoptosis, p53, and Tumor Cell Sensitivity to Anticancer Agents." Cancer Res **59**(7): 1391-1399.

Burczynski, M. E., H. K. Lin, et al. (1999). "Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase." Cancer Res **59**(3): 607-614.

Calin, G. A., C. D. Dumitru, et al. (2002). "Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proc Natl Acad Sci U S A **99**(24): 15524-15529.

Carrington, J. C. and V. Ambros (2003). "Role of microRNAs in plant and animal development." Science **301**(5631): 336-338.

Cavallaro, U. and G. Christofori (2004). "Cell adhesion and signalling by cadherins and Ig-CAMs in cancer." Nat Rev Cancer **4**(2): 118-132.

Chandra, D., S. B. Bratton, et al. (2006). "Intracellular nucleotides act as critical prosurvival factors by binding to cytochrome C and inhibiting apoptosome." Cell **125**(7): 1333-1346.

Chen, Y. J., C. C. Yuan, et al. (2005). "Overexpression of dihydrodiol dehydrogenase is associated with cisplatin-based chemotherapy resistance in ovarian cancer patients." Gynecol Oncol **97**(1): 110-117.

Cheok, M. H. and W. E. Evans (2006). "Acute lymphoblastic leukaemia: a model for the pharmacogenomics of cancer therapy." Nat Rev Cancer **6**(2): 117-129.

Cho, S. G., Y. H. Lee, et al. (2001). "Glutathione S-transferase mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1." J Biol Chem **276**(16): 12749-12755.

Ciaccio, P. J., J. E. Stuart, et al. (1993). "Overproduction of a 37.5-kDa cytosolic protein structurally related to prostaglandin F synthase in ethacrynic acid-resistant human colon cells." Mol Pharmacol **43**(6): 845-853.

Cimmino, A., G. A. Calin, et al. (2005). "miR-15 and miR-16 induce apoptosis by targeting BCL2." Proc Natl Acad Sci U S A **102**(39): 13944-13949.

Circu, M. L. and T. Y. Aw (2008). "Glutathione and apoptosis." Free Radic Res **42**(8): 689-706.

Clark, A. G., G. L. Dick, et al. (1984). "Kinetic studies on a glutathione S-transferase from the larvae of Costelytra zealandica." Biochem J **217**(1): 51-58.

Cooper, D. N. (2002). "Galectinomics: finding themes in complexity." Biochim Biophys Acta **1572**(2-3): 209-231.

Cooper, D. N. and S. H. Barondes (1999). "God must love galectins; he made so many of them." Glycobiology **9**(10): 979-984.

Cory, S. and J. M. Adams (2002). "The Bcl2 family: regulators of the cellular life-or-death switch." Nat Rev Cancer **2**(9): 647-656.

Cowan, K. H. and J. Jolivet (1984). "A methotrexate-resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates." J Biol Chem **259**(17): 10793-10800.

D'Alessio, M., M. De Nicola, et al. (2005). "Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis." FASEB J **19**(11): 1504-1506.

da Silva, C. P., C. R. de Oliveira, et al. (1996). "Apoptosis as a mechanism of cell death induced by different chemotherapeutic drugs in human leukemic T-lymphocytes." Biochem Pharmacol **51**(10): 1331-1340.

Dalle-Donne, I., D. Giustarini, et al. (2005). "S-glutathionylation in human platelets by a thiol-disulfide exchange-independent mechanism." Free Radic Biol Med **38**(11): 1501-1510.

Deng, H. (2006). "Characterization of the reaction products of cytochrome c with glutathione by mass spectrometry." Biochem Biophys Res Commun **342**(1): 73-80.

Deng, H. B., M. Adikari, et al. (2004). "Ubiquitous induction of resistance to platinum drugs in human ovarian, cervical, germ-cell and lung carcinoma tumor cells overexpressing isoforms 1 and 2 of dihydrodiol dehydrogenase." Cancer Chemother Pharmacol **54**(4): 301-307.

Ding, B. C., T. L. Witt, et al. (2001). "Association of deletions and translocation of the reduced folate carrier gene with profound loss of gene expression in methotrexate-resistant K562 human erythroleukemia cells." Biochem Pharmacol **61**(6): 665-675.

Donato, R. (2001). "S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles." Int J Biochem Cell Biol **33**(7): 637-668.

Donato, R. (2003). "Intracellular and extracellular roles of S100 proteins." Microsc Res Tech **60**(6): 540-551.

Dukhanina, E. A., A. S. Dukhanin, et al. (1997). "Spectral studies on the calcium-binding properties of Mts1 protein and its interaction with target protein." FEBS Lett **410**(2-3): 403-406.

Edmonds, B. T., J. Wyckoff, et al. (1996). "Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma." J Cell Sci **109 (Pt 11)**: 2705-2714.

el Alaoui, S., J. Lawry, et al. (1997). "The cell cycle and induction of apoptosis in a hamster fibrosarcoma cell line treated with anti-cancer drugs: its importance to solid tumour chemotherapy." J Neurooncol **31**(1-2): 195-207.

Evan, G. I. and K. H. Vousden (2001). "Proliferation, cell cycle and apoptosis in cancer." Nature **411**(6835): 342-348.

Ferreri, A. J., S. Dell'Oro, et al. (2004). "Aberrant methylation in the promoter region of the reduced folate carrier gene is a potential mechanism of resistance to methotrexate in primary central nervous system lymphomas." Br J Haematol **126**(5): 657-664.

Fisher, D. E. (1994). "Apoptosis in cancer therapy: crossing the threshold." Cell **78**(4): 539-542.

Forget, M. A., S. Turcotte, et al. (2007). "The Wnt pathway regulator DKK1 is preferentially expressed in hormone-resistant breast tumours and in some common cancer types." Br J Cancer **96**(4): 646-653.

Fratelli, M., H. Demol, et al. (2002). "Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes." Proc Natl Acad Sci U S A **99**(6): 3505-3510.

Frixen, U. H., J. Behrens, et al. (1991). "E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells." J Cell Biol **113**(1): 173-185.

Fujiwara, H., N. Arima, et al. (2002). "Clinical significance of serum neuron-specific enolase in patients with adult T-cell leukemia." Am J Hematol **71**(2): 80-84.

Geiger, T., M. Muller, et al. (1998). "Antitumor activity of a PKC-alpha antisense oligonucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted into nude mice." Anticancer Drug Des **13**(1): 35-45.

Ghibelli, L., S. Coppola, et al. (1999). "Glutathione depletion causes cytochrome c release even in the absence of cell commitment to apoptosis." FASEB J **13**(14): 2031-2036.

Giaccia, A. J. and M. B. Kastan (1998). "The complexity of p53 modulation: emerging patterns from divergent signals." Genes Dev **12**(19): 2973-2983.

Gorlick, R., E. Goker, et al. (1997). "Defective transport is a common mechanism of acquired methotrexate resistance in acute lymphocytic leukemia and is associated with decreased reduced folate carrier expression." Blood **89**(3): 1013-1018.

Grassi, G., B. Scaggiante, et al. (2007). "The expression levels of the translational factors eEF1A 1/2 correlate with cell growth but not apoptosis in hepatocellular carcinoma cell lines with different differentiation grade." Biochimie **89**(12): 1544-1552.

Gregory, C. A., H. Singh, et al. (2003). "The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow." J Biol Chem **278**(30): 28067-28078.

Guo, W., J. H. Healey, et al. (1999). "Mechanisms of methotrexate resistance in osteosarcoma." Clin Cancer Res **5**(3): 621-627.

Hancock, J. T., R. Desikan, et al. (2001). "Does the redox status of cytochrome C act as a fail-safe mechanism in the regulation of programmed cell death?" Free Radic Biol Med **31**(5): 697-703.

Hassell, J. A. and D. L. Engelhardt (1976). "The regulation of protein synthesis in animal cells by serum factors." Biochemistry **15**(7): 1375-1381.

Hattangadi, D. K., G. A. DeMasters, et al. (2004). "Influence of p53 and caspase 3 activity on cell death and senescence in response to methotrexate in the breast tumor cell." Biochem Pharmacol **68**(9): 1699-1708.

Hayashita, Y., H. Osada, et al. (2005). "A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation." Cancer Res **65**(21): 9628-9632.

Hayes, J. D., J. U. Flanagan, et al. (2004). "GLUTATHIONE TRANSFERASES." Annual Review of Pharmacology and Toxicology **45**(1): 51-88.

Hayes, J. D. and L. I. McLellan (1999). "Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress." Free Radic Res **31**(4): 273-300.

Hayes, J. D. and D. J. Pulford (1995). "The Glut athione S-Transferase Supergene Family: Regulation of GST and the Contribution of the lsoenzymes to Cancer Chemoprotection and Drug Resistance Part I." Critical Reviews in Biochemistry and Molecular Biology **30**(6): 445-520.

Ho, C. C., P. H. Huang, et al. (2002). "Up-regulated caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation." Am J Pathol **161**(5): 1647-1656.

Hryniuk, W. M. and J. R. Bertino (1969). "Rationale for the selection of chemotherapeutic agents." Adv Intern Med **15**: 267-297.
Hsu, N. Y., H. C. Ho, et al. (2001). "Overexpression of dihydrodiol dehydrogenase as a prognostic marker of non-small cell lung cancer." Cancer Res **61**(6): 2727-2731.
Huang, D. C. and A. Strasser (2000). "BH3-Only proteins-essential initiators of apoptotic cell death." Cell **103**(6): 839-842.
Hung, J. J., K. C. Chow, et al. (2006). "Expression of dihydrodiol dehydrogenase and resistance to chemotherapy and radiotherapy in adenocarcinoma cells of lung." Anticancer Res **26**(4B): 2949-2955.
Iorio, M. V., M. Ferracin, et al. (2005). "MicroRNA gene expression deregulation in human breast cancer." Cancer Res **65**(16): 7065-7070.
Isonishi, S., K. Ohkawa, et al. (2000). "Depletion of protein kinase C (PKC) by 12-O-tetradecanoylphorbol-13-acetate (TPA) enhances platinum drug sensitivity in human ovarian carcinoma cells." Br J Cancer **82**(1): 34-38.
Jez, J. M., T. G. Flynn, et al. (1997). "A new nomenclature for the aldo-keto reductase superfamily." Biochem Pharmacol **54**(6): 639-647.
Johnsson, A., I. Zeelenberg, et al. (2000). "Identification of genes differentially expressed in association with acquired cisplatin resistance." Br J Cancer **83**(8): 1047-1054.
Jolivet, J., K. H. Cowan, et al. (1983). "The Pharmacology and Clinical Use of Methotrexate." New England Journal of Medicine **309**(18): 1094-1104.
Kagan, V. E., V. A. Tyurin, et al. (2005). "Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors." Nat Chem Biol **1**(4): 223-232.
Karnak, D., S. Beder, et al. (2005). "Neuron-specific enolase and lung cancer." Am J Clin Oncol **28**(6): 586-590.
Katula, K. S., A. N. Heinloth, et al. (2007). "Folate deficiency in normal human fibroblasts leads to altered expression of genes primarily linked to cell signaling, the cytoskeleton and extracellular matrix." J Nutr Biochem 18(8): 541-552.
Kaufmann, S. H. (1989). "Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note." Cancer Res **49**(21): 5870-5878.
Kaufmann, S. H. and W. C. Earnshaw (2000). "Induction of apoptosis by cancer chemotherapy." Exp Cell Res **256**(1): 42-49.
Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-257.
Kim, R., M. Emi, et al. (2005). "Caspase-dependent and -independent cell death pathways after DNA damage (Review)." Oncol Rep **14**(3): 595-599.
Kimura, E., K. Nishimura, et al. (2004). "Methotrexate differentially affects growth of suspension and adherent cells." Int J Biochem Cell Biol **36**(5): 814-825.
Kitakata, H., K. Yasumoto, et al. (2007). "A case of primary small cell carcinoma of the breast." Breast Cancer **14**(4): 414-419.
Koch, A., A. Waha, et al. (2005). "Elevated expression of Wnt antagonists is a common event in hepatoblastomas." Clin Cancer Res **11**(12): 4295-4304.
Krishnaswamy, S., Q. Hao, et al. (2005). "UDP glucuronosyltransferase (UGT) 1A6 pharmacogenetics: I. Identification of polymorphisms in the 5'-regulatory and exon 1 regions, and association with human liver UGT1A6 gene expression and glucuronidation." J Pharmacol Exp Ther **313**(3): 1331-1339.
Kuwana, T., M. R. Mackey, et al. (2002). "Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane." Cell **111**(3): 331-342.
Lahn, M., G. Kohler, et al. (2004). "Protein kinase C alpha expression in breast and ovarian cancer." Oncology **67**(1): 1-10.
Liani, E., L. Rothem, et al. (2003). "Loss of folylpoly-gamma-glutamate synthetase activity is a dominant mechanism of resistance to polyglutamylation-dependent novel antifolates in multiple human leukemia sublines." Int J Cancer **103**(5): 587-599.
Livingstone, L. R., A. White, et al. (1992). "Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53." Cell **70**(6): 923-935.
Lloyd, B. H., A. Platt-Higgins, et al. (1998). "Human S100A4 (p9Ka) induces the metastatic phenotype upon benign tumour cells." Oncogene **17**(4): 465-473.
Ma, J., C. Dong, et al. (2010). "MicroRNA and drug resistance." Cancer Gene Ther **17**(8): 523-531.

Mahon, P. C., P. Baril, et al. (2007). "S100A4 contributes to the suppression of BNIP3 expression, chemoresistance, and inhibition of apoptosis in pancreatic cancer." Cancer Res **67**(14): 6786-6795.

Martelli, A. M., N. Sang, et al. (1999). "Multiple biological responses activated by nuclear protein kinase C." J Cell Biochem **74**(4): 499-521.

Martin, M. C., L. A. Allan, et al. (2005). "Protein kinase A regulates caspase-9 activation by Apaf-1 downstream of cytochrome c." J Biol Chem **280**(15): 15449-15455.

Maser, E. (1995). "Xenobiotic carbonyl reduction and physiological steroid oxidoreduction. The pluripotency of several hydroxysteroid dehydrogenases." Biochem Pharmacol **49**(4): 421-440.

Mauritz, R., G. J. Peters, et al. (2002). "Multiple mechanisms of resistance to methotrexate and novel antifolates in human CCRF-CEM leukemia cells and their implications for folate homeostasis." Biochem Pharmacol **63**(2): 105-115.

McCloskey, D. E., J. J. McGuire, et al. (1991). "Decreased folylpolyglutamate synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines." J Biol Chem **266**(10): 6181-6187.

Mencia, N., E. Selga, et al. (2011). "Underexpression of miR-224 in methotrexate resistant human colon cancer cells." Biochem Pharmacol.

Metzler, M., M. Wilda, et al. (2004). "High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma." Genes Chromosomes Cancer **39**(2): 167-169.

Monaghan, A. P., P. Kioschis, et al. (1999). "Dickkopf genes are co-ordinately expressed in mesodermal lineages." Mech Dev **87**(1-2): 45-56.

Murakami, Y., T. Yasuda, et al. (2006). "Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues." Oncogene **25**(17): 2537-2545.

Narita, M., S. Shimizu, et al. (1998). "Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria." Proc Natl Acad Sci U S A **95**(25): 14681-14686.

O'Connor, T., L. S. Ireland, et al. (1999). "Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members." Biochem J **343 Pt 2**: 487-504.

Oka, N., M. Yamamoto, et al. (1997). "Caveolin interaction with protein kinase C. Isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide." J Biol Chem **272**(52): 33416-33421.

Okouchi, M., N. Okayama, et al. (2006). "NRF2-dependent glutamate-L-cysteine ligase catalytic subunit expression mediates insulin protection against hyperglycemia- induced brain endothelial cell apoptosis." Curr Neurovasc Res **3**(4): 249-261.

Ott, M., V. Gogvadze, et al. (2007). "Mitochondria, oxidative stress and cell death." Apoptosis **12**(5): 913-922.

Pan, Z., D. W. Voehringer, et al. (1999). "Analysis of redox regulation of cytochrome c-induced apoptosis in a cell-free system." Cell Death Differ **6**(7): 683-688.

Parker, C., P. A. Whittaker, et al. (1994). "Induction of 18A2/mts1 gene expression and its effects on metastasis and cell cycle control." DNA Cell Biol **13**(10): 1021-1028.

Penning, T. M. (2005). "AKR1B10: a new diagnostic marker of non-small cell lung carcinoma in smokers." Clin Cancer Res **11**(5): 1687-1690.

Perl, A. K., P. Wilgenbus, et al. (1998). "A causal role for E-cadherin in the transition from adenoma to carcinoma." Nature **392**(6672): 190-193.

Pias, E. K., O. Y. Ekshyyan, et al. (2003). "Differential effects of superoxide dismutase isoform expression on hydroperoxide-induced apoptosis in PC-12 cells." J Biol Chem **278**(15): 13294-13301.

Robertson, J. D., S. Orrenius, et al. (2000). "Review: nuclear events in apoptosis." J Struct Biol **129**(2-3): 346-358.

Romero, M. F., M. A. Hediger, et al. (1997). "Expression cloning and characterization of a renal electrogenic Na+/HCO3- cotransporter." Nature **387**(6631): 409-413.

Rosowsky, A., J. E. Wright, et al. (1982). "Methotrexate analogues. 15. A methotrexate analogue designed for active-site-directed irreversible inactivation of dihydrofolate reductase." J Med Chem **25**(8): 960-964.

Rothbacher, U. and P. Lemaire (2002). "Creme de la Kremen of Wnt signalling inhibition." Nat Cell Biol **4**(7): E172-173.

Rothem, L., I. Ifergan, et al. (2002). "Resistance to multiple novel antifolates is mediated via defective drug transport resulting from clustered mutations in the reduced folate carrier gene in human leukaemia cell lines." Biochem J **367**(Pt 3): 741-750.

Roy, K., J. H. Chiao, et al. (1998). "Chromosomal localization of the murine RFC-1 gene encoding a folate transporter and its amplification in an antifolate resistant variant overproducing the transporter." Cancer Genet Cytogenet **105**(1): 29-38.

Roy, K., M. G. Egan, et al. (1997). "Posttranscriptionally mediated decreases in folylpolyglutamate synthetase gene expression in some folate analogue-resistant variants of the L1210 cell. Evidence for an altered cognate mRNA in the variants affecting the rate of de novo synthesis of the enzyme." J Biol Chem **272**(11): 6903-6908.

Selga, E., C. Morales, et al. (2008). "Role of caveolin 1, E-cadherin, Enolase 2 and PKCalpha on resistance to methotrexate in human HT29 colon cancer cells." BMC Med Genomics **1**: 35.

Selga, E., V. Noe, et al. (2008). "Transcriptional regulation of aldo-keto reductase 1C1 in HT29 human colon cancer cells resistant to methotrexate: role in the cell cycle and apoptosis." Biochem Pharmacol **75**(2): 414-426.

Selga, E., C. Oleaga, et al. (2009). "Networking of differentially expressed genes in human cancer cells resistant to methotrexate." Genome Med **1**(9): 83.

Sharma, R. C., Y. G. Assaraf, et al. (1991). "A phenotype conferring selective resistance to lipophilic antifolates in Chinese hamster ovary cells." Cancer Res **51**(11): 2949-2959.

Sharom, F. J. (1997). "The P-glycoprotein efflux pump: how does it transport drugs?" J Membr Biol **160**(3): 161-175.

Sharom, F. J. (1997). "The P-glycoprotein multidrug transporter: interactions with membrane lipids, and their modulation of activity." Biochem Soc Trans **25**(3): 1088-1096.

Shelton, M. D., P. B. Chock, et al. (2005). "Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation." Antioxid Redox Signal **7**(3-4): 348-366.

Shen, H., L. Kauvar, et al. (1997). "Importance of glutathione and associated enzymes in drug response." Oncol Res **9**(6-7): 295-302.

Shepherd, J. C., U. Walldorf, et al. (1989). "Fruit flies with additional expression of the elongation factor EF-1 alpha live longer." Proc Natl Acad Sci U S A **86**(19): 7520-7521.

Sherbet, G. V. and M. S. Lakshmi (1998). "S100A4 (MTS1) calcium binding protein in cancer growth, invasion and metastasis." Anticancer Res **18**(4A): 2415-2421.

Schimke, R. T., P. C. Brown, et al. (1981). "Chromosomal and extrachromosomal localization of amplified dihydrofolate reductase genes in cultured mammalian cells." Cold Spring Harb Symp Quant Biol **45** Pt 2: 785-797

Srimatkandada, S., B. I. Schweitzer, et al. (1989). "Amplification of a polymorphic dihydrofolate reductase gene expressing an enzyme with decreased binding to methotrexate in a human colon carcinoma cell line, HCT-8R4, resistant to this drug." J Biol Chem **264**(6): 3524-3528.

Suto, D., K. Sato, et al. (2005). "Suppression of the pro-apoptotic function of cytochrome c by singlet oxygen via a haem redox state-independent mechanism." Biochem J **392**(Pt 2): 399-406.

Talapatra, S., J. D. Wagner, et al. (2002). "Elongation factor-1 alpha is a selective regulator of growth factor withdrawal and ER stress-induced apoptosis." Cell Death Differ **9**(8): 856-861.

Tannock, I. F. and C. Lee (2001). "Evidence against apoptosis as a major mechanism for reproductive cell death following treatment of cell lines with anti-cancer drugs." Br J Cancer **84**(1): 100-105.

Tatsuka, M., H. Mitsui, et al. (1992). "Elongation factor-1 alpha gene determines susceptibility to transformation." Nature **359**(6393): 333-336.

Thompson, T. C. (1998). "Metastasis-related genes in prostate cancer: the role of caveolin-1." Cancer Metastasis Rev **17**(4): 439-442.

Thornton, S., N. Anand, et al. (2003). "Not just for housekeeping: protein initiation and elongation factors in cell growth and tumorigenesis." J Mol Med (Berl) **81**(9): 536-548.

Torres, V. A., J. C. Tapia, et al. (2007). "E-cadherin is required for caveolin-1-mediated down-regulation of the inhibitor of apoptosis protein survivin via reduced beta-catenin-Tcf/Lef-dependent transcription." Mol Cell Biol **27**(21): 7703-7717.

Twiddy, D., D. G. Brown, et al. (2004). "Pro-apoptotic proteins released from the mitochondria regulate the protein composition and caspase-processing activity of the native Apaf-1/caspase-9 apoptosome complex." J Biol Chem **279**(19): 19665-19682.

Voorhoeve, P. M., C. le Sage, et al. (2006). "A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors." Cell **124**(6): 1169-1181.

Voorzanger-Rousselot, N., D. Goehrig, et al. (2007). "Increased Dickkopf-1 expression in breast cancer bone metastases." Br J Cancer **97**(7): 964-970.

Wang, X. Y. and H. T. Liu (1998). "Antisense expression of protein kinase C alpha improved sensitivity to anticancer drugs in human lung cancer LTEPa-2 cells." Zhongguo Yao Li Xue Bao **19**(3): 265-268.

Wang, X. Y., E. Repasky, et al. (1999). "Antisense inhibition of protein kinase Calpha reverses the transformed phenotype in human lung carcinoma cells." Exp Cell Res **250**(1): 253-263.

Weeks, R., W. Dowhan, et al. (1997). "Isolation and expression of an isoform of human CDP-diacylglycerol synthase cDNA." DNA Cell Biol **16**(3): 281-289.

Yang, G., L. D. Truong, et al. (1998). "Elevated expression of caveolin is associated with prostate and breast cancer." Clin Cancer Res **4**(8): 1873-1880.

Yeh, C. S., J. Y. Wang, et al. (2008). "Significance of the glycolytic pathway and glycolysis related-genes in tumorigenesis of human colorectal cancers." Oncol Rep **19**(1): 81-91.

Yu, G., S. Ahmad, et al. (1991). "Transfection with protein kinase C alpha confers increased multidrug resistance to MCF-7 cells expressing P-glycoprotein." Cancer Commun **3**(6): 181-189.

Zhao, R., F. Gao, et al. (2004). "A prominent low-pH methotrexate transport activity in human solid tumors: contribution to the preservation of methotrexate pharmacologic activity in HeLa cells lacking the reduced folate carrier." Clin Cancer Res **10**(2): 718-727.

Zhao, R. and I. D. Goldman (2003). "Resistance to antifolates." Oncogene **22**(47): 7431-7457.

Zou, H., Y. Li, et al. (1999). "An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9." J Biol Chem **274**(17): 11549-11556.

Objectives

The main objective of the present work is to assess the potential involvement of the apoptotic regulation by cytochrome c redox state in the development of the resistance to MTX in human breast cancer cells. As described above, drug resistance is a complex process in which several pathways can be involved. In this study we propose a new model involving the cytochrome c redox state in regulation of MTX-dependent apoptosis. To achieve that, we firstly evaluate the effect of cytochrome c redox-state in sensitivity to MTX and in MTX-dependent apoptotic effect using cytochrome c- reducer agents such as TMPD, ascorbate or reduced GSH.

GSTs and GSH have been associated to drug-resistance mechanisms. The overexpression of some cytosolic GSTs isoforms in MCF-7 breast cancer cells can be the link between the role of the cytochrome c redox-state in apoptotic regulation and MTX-resistance. We aim to find out their roles in redox-state of cytochrome c and its contribution to MTX resistance in human cancer cells.

Chapter II - The redox state of cytochrome c modulates resistance to methotrexate in human breast cancer

This chapter was submitted to an ISI journal in 05/12/2012 as:

Barros, S., Mencía N., Santos, C., Noé, V. & Ciudad, C.J. (2012). The redox state of cytochrome c modulates resistance to methotrexate in human breast cancer. PLOS One 2012

1. Abstract

1.1. Background

Methotrexate is a chemotherapeutic agent used to treat a variety of cancers. However, the occurrence of resistance limits its effectiveness. Cytochrome c in its reduced state is less capable of triggering the apoptotic cascade. Thus, we set up to study the relationship among redox state of cytochrome c, apoptosis and the development of resistance to methotrexate in human breast cancer cells.

1.2. Results

Cell incubation with cytochrome c-reducing agents, such as tetramethylphenylenediamine, ascorbate or reduced glutathione, decreased the mortality and apoptosis triggered by methotrexate. Conversely, depletion of glutathione increased the apoptotic action of methotrexate, showing an involvement of cytochrome c redox state in methotrexate-induced apoptosis. Methotrexate-resistant cells showed increased levels of endogenous reduced glutathione and a higher capability to reduce exogenous cytochrome c. Using functional genomics we detected the overexpression of *GSTM1* and *GSTM4* in methotrexate-resistant MCF7 breast cancer cells.The inhibition of these isoforms of GSTM caused an increase in methotrexate cytotoxicity in sensitive cells.

1.3. Conclusions

We conclude that overexpression of specific GSTMs, *GSTM1* and *GSTM4*, together with increased endogenous reduced glutathione levels help to maintain a more reduced state of cytochrome c which, in turn, would decrease apoptosis, thus contributing to methotrexate resistance in human cancer cells.

2. Introduction

Methotrexate (MTX) is a chemotherapeutic agent widely used, alone or in combination with other chemotherapeutic agents, for the treatment of a range of cancers, such as breast cancer, osteosarcoma, head and neck cancer, lymphoma and acute lymphoblastic leukemia (Jolivet et al. 1983). As a structural analogue of folic acid, MTX is a high affinity inhibitor of dihydrofolate reductase (DHFR) by competing with dihydrofolate for the active site. DHFR catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate involved in the biosynthesis of thymidylate, hypoxantine and glycine, needed for DNA synthesis (Blakley and Cocco 1984; Noe et al. 1995; Chu et al. 1996). Once DHFR is inhibited by MTX, there is suppression of DNA synthesis and cell proliferation is affected. However, the main drawback of using MTX in cancer therapy is the occurrence of resistance upon treatment, thus compromising its effectiveness. Several MTX resistance mechanisms have been described such as gene amplification of the *DHFR* locus (Alt et al. 1978; Sirotnak et al. 1981), deficiency in MTX transport (Gorlick, Goker et al. 1997; Liani et al. 2003) or MTX polyglutamation (Haber et al. 1981), expression of the MDR phenotype and mutations of the target (DHFR protein) (Selga et al. 2008). Altered gene or miRNA expression also contribute to MTX resistance such as increases in *AKR1C1* (Mencia et al. 2010), S100A4 (Selga et al. 2008), caveolin-1, enolase-2, *PRKCA* (Selga et al. 2009), *DKK1*, *EEF1A1*, and UGT1A family (de Almagro et al. 2011; Mencia et al. 2011), and the decrease of E-Cadherin-1 (Selga et al. 2009) or miR-224 (Mencia et al. 2011).

Reduced glutathione (GSH) and Glutathione S-transferases (GSTs) have been implicated in the development of drug resistance in cancer chemotherapy (Tew 1994; McLellan and Wolf 1999). The GSTs enzymatic family belongs to a Phase II detoxification program functioning as a cellular protection from attack by reactive electrophiles associated to environmental stresses and drugs (Townsend and Tew 2003). This family is mainly responsible for the conjugation of GSH to electrophilic compounds and includes three main types, cytosolic, mitochondrial and membrane-bound microsomal. Cytosolic GSTs are divided into seven classes: alpha (A), Mu (M), Omega (O), Pi (P), Sigma (S), Theta (T) and Zeta (Z) [20-23]. GSTs are thought to be involved in the development of drug resistance via direct

detoxification or by regulation of the MAP kinase pathway, specifically JNK-pathway as reviewed in (Townsend and Tew 2003).

Cytochrome c is a heme-protein bound to the mitochondrial inner membrane by an interaction with the anionic phospholipid cardiolipin, which keeps cytochrome c in its proper location and prevents its release to the cytosol (Ott et al. 2007). Under physiological conditions, cytochrome c is responsible for the electron transfer between complexes III and IV of the mitochondrial electron transport chain whereas under oxidative stress, the peroxidase activity of cytochrome c is activated, cardiolipin becomes peroxidized, and loses its affinity for cytochrome c allowing its release to the cytosol (Kagan et al. 2005; Belikova et al. 2006). Once in the cytosol, cytochrome c can induce apoptosis only in its oxidized form (Hancock et al. 2001; Suto et al. 2005; Borutaite and Brown 2007; Brown and Borutaite 2008; Li et al. 2008). The presence of high levels of cytosolic GSH holds the released cytochrome c inactive in a reduced state, thus preventing the progression of the apoptotic cascade (Hancock et al. 2003; Vaughn and Deshmukh 2008).

In this study, we investigated the effect of the reduction state of cytochrome c on MTX sensitivity and apoptosis and its relationship with the different GSTs overexpressed in breast cancer cells resistant to MTX, to evaluate a possible connection between GSTs and GSH in the reduction state of cytochrome c and the development of MTX resistance.

3. Materials and Methods

3.1. Cell lines and cell culture

Human breast cancer MCF7 and 10^{-6}M MTX-resistant MCF7 (MCF7-R) cell lines were used. Resistant cells were obtained previously in the laboratory upon incubation with stepwise concentrations of MTX (Almirall, Barcelona, Spain) as described in (Selga et al. 2008). In all experimental procedures, cells were grown in Ham's F12 medium lacking the final products of DHFR activity, glycine, hypoxanthine and thymidine (-GHT), supplemented with 7% v/v dialyzed fetal bovine serum (GIBCO, Life Technologies, Madrid, Spain), 14mM sodium

bicarbonate (1.176g/l), penicillin G (100U/ml) and streptomycin (100mg/l). Cells were maintained at 37ºC in a humidified atmosphere of 5% CO2 in air. Before reaching 70% confluence, cells were sub-cultured by treatment with 0.05% trypsin in PBS 1X. All these components were purchased from Sigma-Aldrich (Madrid, Spain).

3.2. Cell viability assay

Cell viability was assessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann 1983) in 6-well dishes. Cells were incubated with 0.25% MTT and 270μM of sodium succinate (Sigma-Aldrich, Madrid, Spain) and allowed to react for 2h at 37ºC. The medium was removed and 1ml of the solubilization reagent (0.57% acetic acid and 10% SDS (Sodium Dodecyl Sulfate) in DMSO (dimethyl sulfoxide) was added (Applichem, Ecogen, Barcelona, Spain). Cell viability was measured at 570nm in a WPA S2100 Diode Array Spectrophotometer. The results were expressed as percentage of cell survival relative to the control (untreated cells).

3.3. Microarrays

Gene expression was analyzed by hybridization to GeneChip® Human Genome U133 PLUS 2.0 microarrays from Affymetrix, containing 54,675 transcripts and variants. Total RNA for cDNA arrays was prepared from triplicate samples from both control and resistant cells using the RNAeasy Mini kit (Qiagen, Madrid, Spain) following the recommendations of the manufacturer. The integrity of the RNA species was checked using the Bioanalyzer 2100 system (Agilent, Madrid, Spain). Labeling, hybridization and detection were carried out following the manufacturer's specifications.

3.4. Microarrays data analysis

Quantification was carried out with GeneSpring GX 12.0 software (Agilent, Madrid, Spain), which allows multi-filter comparisons using data from different

experiments to perform the normalization, generation of lists and the functional classification of the differentially expressed genes. The input data was subjected to preprocess baseline transformation using the RMA summarization algorithm using the median of control samples. After grouping the triplicates of each experimental condition, lists of differentially expressed genes could be generated by using volcano plot analysis. T-test unpaired was applied using asymptotic p-value computation and multiple testing correction of Benjamini-Hochberg false discovery rate, FDR. The expression of each gene was reported as the ratio of the value obtained for the resistant condition relative to the control condition after normalization and statistical analysis of the data. The corrected p-value cut-off applied was of <0.05; then the output of this statistical analysis was filtered by fold expression, selecting specifically those genes that had a differential expression of at least 2-fold.

3.5. RT-Real-Time PCR

Total RNA was extracted using the UltraspecTM RNA reagent (Biotecx, Ecogen, Barcelona, Spain) following the manufacturer's instructions. Complementary DNA (cDNA) was synthetized in a total volume of 20µl by mixing 1µg of total RNA, 125ng of random hexamers (Roche, Mannheim, Germany), in the presence of 75mM KCl, 3mM $MgCl_2$, 50mM Tris-HCl buffer, pH 8.3, 10mM dithiothreitol (Invitrogen, Life Technologies, Madrid, Spain), 20 units of RNAsin (Promega, Madrid, Spain), 0.5mM dNTPs (Applichem, Ecogen, Barcelona, Spain) and 200 units of MLV-reverse transcriptase (Invitrogen, Life Technologies, Madrid, Spain). The reaction mix was incubated at 37ºC for 1h and the cDNA product was used for subsequent amplification.

Gene expression levels were quantified by SYBR Green RT-Real Time PCR reaction in a final volume of 20µl with specific forward and reverse primers, using the StepOnePlusTM detection system (Applied Biosystems, Life Technologies, Madrid, Spain). The sequences of the forward and reverse primers (Sigma-Aldrich, Madrid, Spain) are given in Table 2.

Changes in gene expression were calculated using the quantitative ΔΔCt method and normalized against Hypoxanthine-phosphoribosyl transferase (HPRT) in each sample.

Table 2 – Primer sequences. The sequences for the forward and reverse primer for detection of *GSTM1*, *GSTM4* and HPRT mRNA levels used for RT-Real Time PCR are given.

Gene	Forward (5'-3')	Reverse (5'-3')
GSTM1	TGAAGCCTCAGCTACCCACT	AACCAGTCAATGCTGCTCCT
GSTM4	TTTCCTCGCCTATGATGTCC	GCTGAGTATGGGCTCCTCAC
HPRT	TGCTCGAGATGTGATGAAGG	TCCCCTGTTGACTGGTCATT

3.6. Inhibition of *GSTM1* and *GSTM4* expression levels

GSTM1 and *GSTM4* expression was inhibited by specific PPRH-hairpins (hp), hpGSTM1 and hpGSTM4, respectively (de Almagro et al. 2009; de Almagro et al. 2011). The Triplex-Forming Oligonucleotide Target Sequence Search software (M.D. Anderson Cancer Center, Houston, TX) (spi.mdanderson.org/tfo/) was used to design the hairpins. BLAST analyses were performed to check for the specificity of each sequence. Cells were plated in 6-well dishes in 1 ml of medium the night before transfection and each hairpin was mixed with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Roche, Mannheim, Germany) at the appropriate oligonucleotide–DOTAP molar ratio (1:100) for 15 min at RT before lipofecting the cells. The sequences of the hairpins (Sigma-Aldrich, Madrid, Spain) are listed in Table 3.

For RNA determination, cells were transfected and total RNA was extracted 30h later. Gene expression was quantified as described above.

For viability assays, cells were incubated with each hairpin for 24h before MTX treatment. Survival was determined after 3 days

Table 3 – Hairpin sequences. The sequences for the polypurine reverse Hoogsteen hairpins used for specific inhibition of *GSTM1, GST*M4, as well as the scrambled negative control are given.

Hairpin	Sequence (5'-3')
hpGSTM1	GAAGGGGAGGGAAGAGAGAAGTTTTTGAAGAGAGAAGGGAGGGGAAG
hpGSTM4	GGAGAAGAAGAAAAGGGGGAAGTTTTTGAAGGGGGAAAAGAAGAAGAGG
hpSC	AAGAGAAAAAGAGAAAGAAGAGAGGGTTTTTGGGAGAGAAGAAAGAGAAAAAGAGAA

3.7. Apoptosis

Apoptosis was determined by the Rhodamine 123/Propidium Iodide assay. Cells ($6x10^4$) were plated in 6-well dishes, and treated with the different agents (TMPD, ascorbate, veratridine) alone or in combination with MTX for the indicated times and concentrations. Then cells were incubated for 30min with 5µl of Rhodamine 123 (1µg/µl). All the cells in each well were harvested and centrifuged at 800 x g for 5min. The cell pellet was washed twice with 1ml of PBS 1X + 1%BSA solution and resuspended in 500µl of PBS 1X + BSA 1% solution containing 0.5µl PI (5µg/µl). The entire procedure was performed at 4ºC. All these reagents were purchased from Sigma-Aldrich (Madrid, Spain). Samples were analyzed by flow cytometry in the Coulter Epics XL™ cytometer (Beckman, Barcelona, Spain) at an excitation wavelength of 488 nm by reading the fluorescence of rhodamine123 at 525 nm. Cells that were negative for both rhodamine123 and PI were counted as the apoptotic population. Summit v4.3 software was used to analyze the data.

3.8. Oxygen consumption assay

Cellular oxygen consumption was monitored polarographically with a Clark-type oxygen electrode using Hansatech Oxygraph Measurement System (Hansatech, Norfolk, UK). The assay was performed using $6x10^4$cells/ml in the presence or in the absence of 5µM TMPD in 1ml of PBS (pH 7.4) as experimental medium at 37ºC. Oxygen consumption was measured either in the absence or the presence of TMPD during 5 min for each condition and determined by the slope calculated directly by the OxygraphPlus Software.

3.9. GSH endogenous levels

Endogenous GSH levels were determined using the Glutathione Assay Kit, Fluorimetric (Sigma-Aldrich®) based on a fluorimetric reaction catalyzed by GSTs between monochlorobimane, a thiol probe, and GSH. Briefly, the assay was performed with $6x10^4$ sensitive and resistant MCF7 cells and the formation of the fluorescent adduct GSH-monochlorobimane was monitored at 390nm for excitation and 478nm for emission during 1h.

3.10. Exogenous cytochrome c reduction by cytosolic extracts

Cytosolic extracts were obtained from MCF7 cells. Cells were collected in ice-cold PBS by scraping and centrifuged at 1500 x g for 10 min. The cell pellet was resuspended in 3ml of lysing buffer prepared according to Borutaite and Brown (2007) and homogenized in Glass/Teflon Potter Elvehjem homogenizer (20 strokes). The homogenate was further centrifuged in the same conditions as above and the supernatant was further centrifuged at 22,000 x g for 30 min and the resulting supernatant corresponds to the cytosolic extract. The entire procedure was performed at 4ºC. The reduction of exogenous cytochrome c by cytosolic extracts (100µg/ml of total protein) was followed spectrophotometrically. The analysis measured the absorbance spectra between 500 and 600nm wavelengths after incubation for 15min at 37ºC of exogenous cytochrome c (10µM) with cytosolic extracts from sensitive or resistant MCF7 cells. Reduction level of cytochrome c was expressed as absorbance at 550 nm minus absorbance at 535 nm and was normalized to the total protein concentration of cytosolic extract used.

3.11. Statistical analysis

Data are presented as the mean ± SE for at least three different experiments. Analyses were performed using SPSS v.18.3 software. Differences with p-value <0.05 were considered significant.

4. Results and Discussion

4.1. Effect of TMPD and ascorbate on the cytotoxicity produced by methotrexate tetramethylphenylenediamine (TMPD) and ascorbate

TMPD and ascorbate have been described as external reductants of cytochrome c both in cytosol and mitochondria (Sarti et al. 1992; Nishimura et al. 2001; Borutaite and Brown 2007). In this direction, we used both agents to study the role of the reduced state of cytochrome c in the sensitivity to methotrexate.

First, the reduction of cytochrome c by TMPD treatment in MCF7 cells was confirmed by determining the changes in O_2 consumption using an oxygraph upon treatment with this chemical reagent. One of the classic end-points to analyze mitochondrial function is to assess the changes in oxygen consumption since O_2 is the ultimate electron acceptor (Pan et al. 1999). This method is commonly used (Clarke et al. 1984; Sarti et al. 1992; Nishimura et al. 2001; Howarth et al. 2006; Ripple et al. 2010), it calculates the variation of O_2 concentration over time and offers the unique advantage of being able to add other components during the experiment. The slope of the graph represents the O_2 consumption rate. As shown in figure 4, TMPD addition increased O_2 consumption as represented by a steeper slope 0.5865 nmol O_2/ml/min compared to basal consumption rate 0.0335 nmol O_2/ml/min.

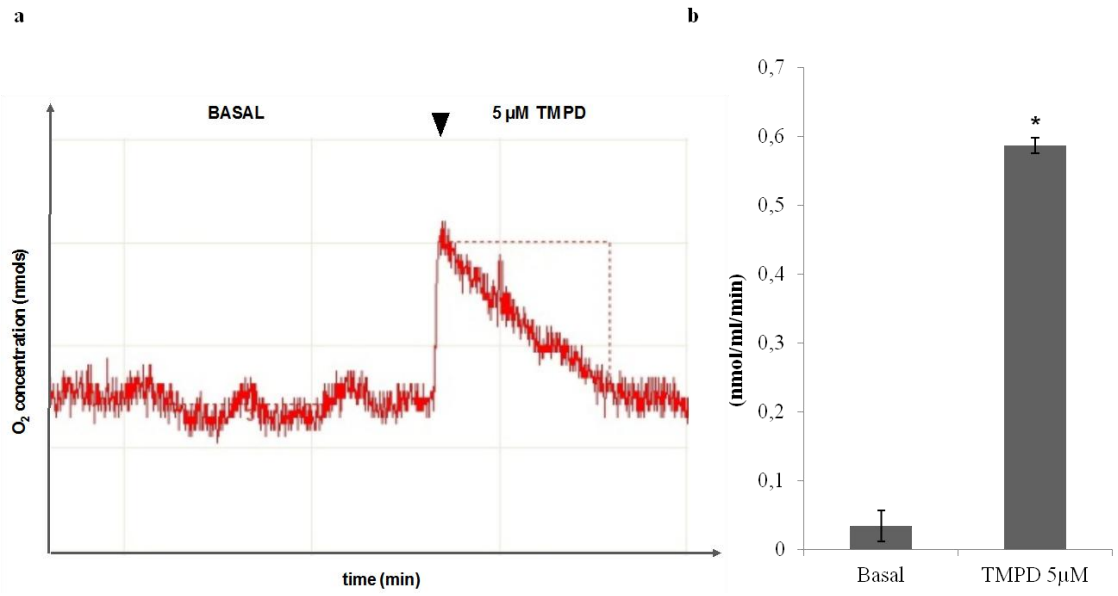


Figure 4 – O_2 consumption analysis upon treatment with TMPD.

Representative image of oxygen concentration determination over time in cell extracts under basal conditions and after treatment with 5µM TMPD (a). The results are expressed as the O_2 consumption rate in each condition and represent mean value ± SE of three independent experiments (b). * $p<0.05$.

MCF7 cells were incubated with 5µM TMPD or 300µM ascorbate, either alone or in combination with 30nM MTX and cell viability was determined after 3 or 6 days, respectively. The incubation with TMPD and ascorbate started 6h before the addition of MTX. The presence of TMPD or ascorbate, which alone did not cause significant cell death, decreased the cytotoxic effect of MTX (Figure 5a, b). The reduction in cytotoxicity was more evident in the presence of TMPD with a recover in cell survival of 26%. The combination of MTX with ascorbate was less effective as the presence of ascorbate only counteracted the action of MTX by 14%. Since the cytotoxic effect of MTX decreased in the presence of these two reducing agents we could hypothesize that the redox state of cytochrome c might be involved in the sensitization of cells to MTX-induced apoptosis.

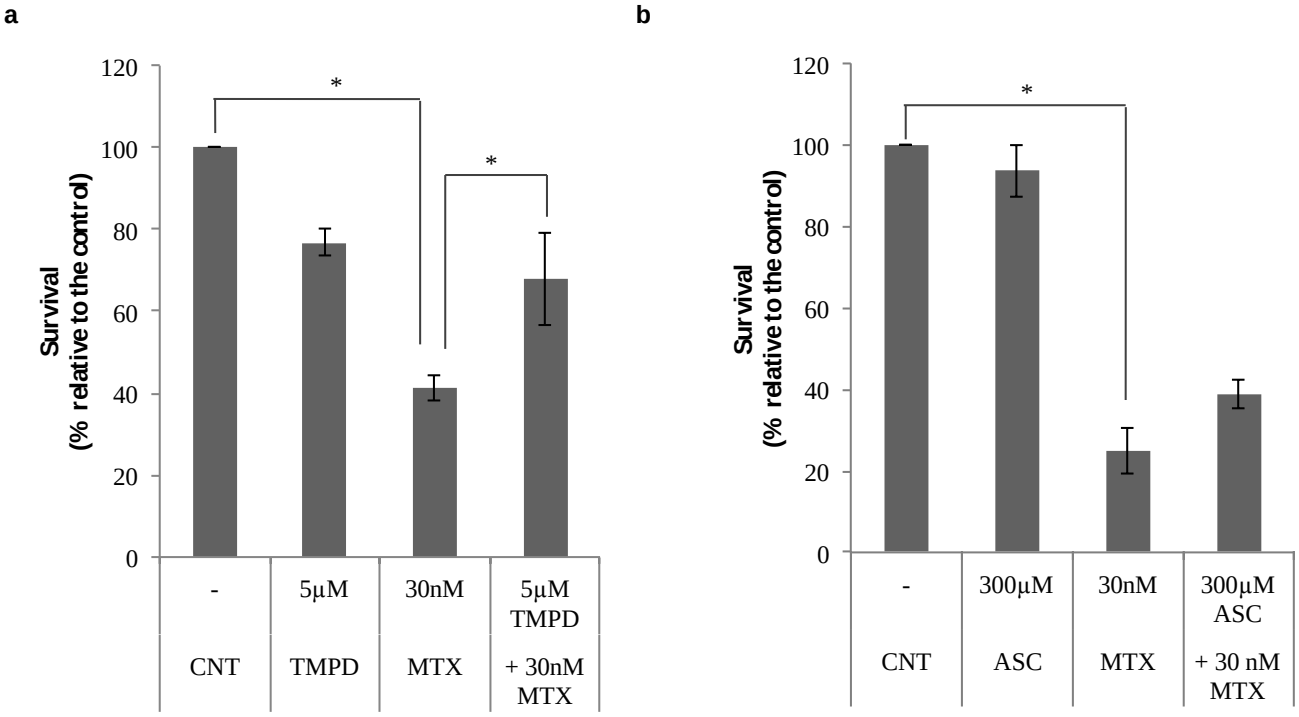


Figure 5 – Effect of TMPD or ascorbate (ASC) in combination with MTX on cell viability. Cells ($6x10^4$) were incubated in 1 ml of medium with TMPD (a) or ASC (b) either alone or in combination with MTX, at the indicated concentrations, for 3 or 6 days, respectively, and cell viability was determined using the MTT assay. Pre-treatment with TMPD or ASC was performed 6h before MTX incubation. Results are expressed as % of surviving cells compared to the control (untreated cells) and represent the mean ± SE of 3 experiments. * $p<0.05$.

4.2. TMPD and ascorbate decrease the apoptotic effect of MTX

It has been demonstrated that MTX can induce apoptosis mediated by cytochrome c release (Pidgeon et al. 2003; Huang et al. 2005). For this reason, we wanted to get further insight into the role of cytochrome c redox state in MTX-induced apoptosis. Levels of apoptosis were determined by the loss of mitochondrial membrane potential (MMP) using the Rhodamine 123/Propidium Iodide assay. Incubation with 50nM or 100nM MTX revealed an increase in apoptosis of 1.65-fold and 1.9-fold, respectively, referred to untreated cells. Treatment with TMPD (Figure 6a) or ascorbate (Figure 6b) before MTX prevented this apoptotic effect by 37% and 20%, respectively. Thus, by reducing cytochrome c with either TMPD or ascorbate, we were able to decrease MTX-induced apoptosis.

Since TMPD was shown to be freely permeable across cytoplasmic and mitochondrial membranes (Sarti et al. 1992) the reduction of cytochrome c could take place in the mitochondria or after its release, as shown by Boturaite and Brown (2007). Regardless the exact mechanism, it is clear from our results that the reduced state of cytochrome c correlates with a lower proapoptotic effect of MTX.

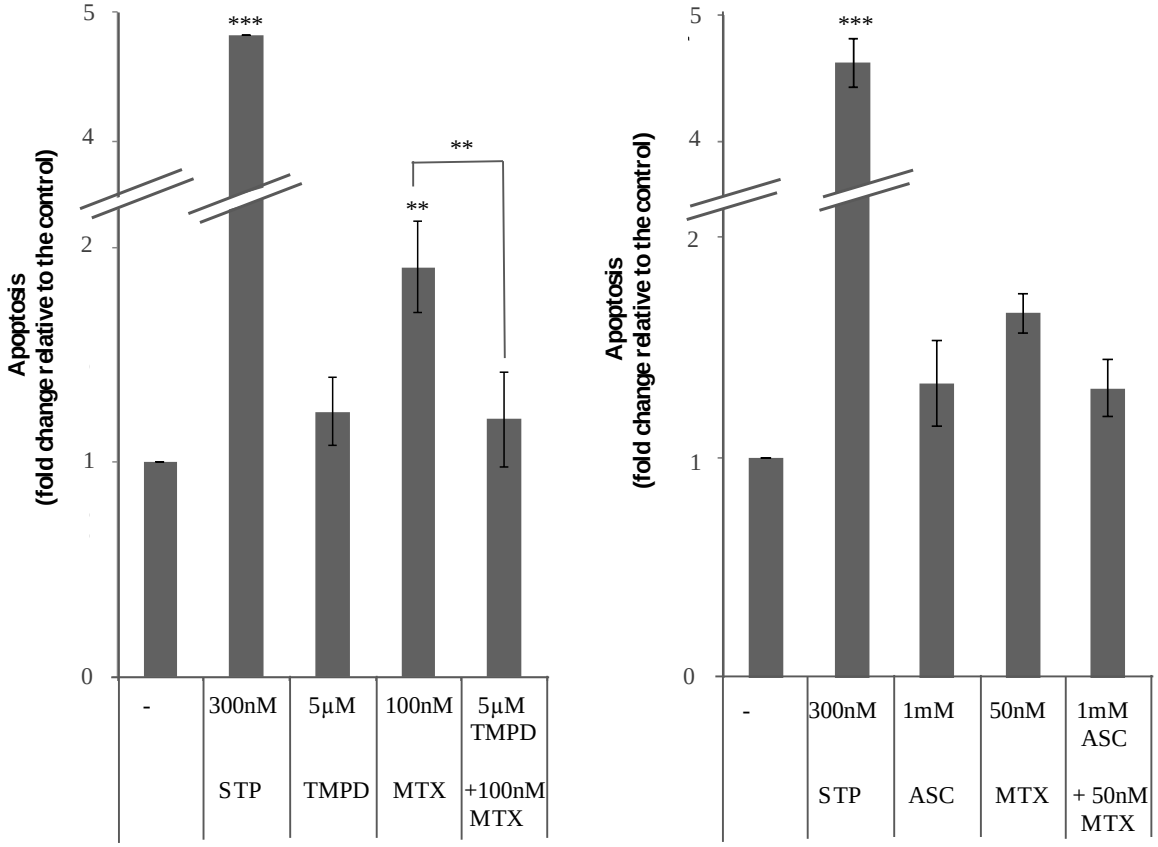


Figure 6 – Effect of TMPD and ascorbate (ASC) on apoptosis induced by MTX. Cells were incubated with TMPD (a) or ASC (b) either alone or in combination with MTX, at the indicated concentrations for 18h. Pre-treatment with TMPD or ascorbate was performed 12h before MTX incubation. Apoptosis was assessed by changes in mitochondrial membrane potential determined using the Rhodamine 123/Propidium Iodide assay and it is expressed as fold change compared to the control and represent the mean ± SE of 3 experiments. *p>0.05, **p>0.01, ***p>0.001.

4.3. Effect of addition or depletion of GSH on MTX action

The redox state of cytochrome c is partially responsible for its apoptotic activity. Our results showed that exogenous reducing agents of cytochrome c were

able to modulate the response towards MTX (figures 5 and 6). It has been described that GSH can reduce cytochrome c (Hancock et al. 2001; Piasentier et al. 2003; Suto et al. 2005; Abdellatif et al. 2007), and therefore we wanted to study whether GSH could exert a role in MTX resistance in MCF7 cells.

GSH is one of the most important regulators of intracellular redox balance, performing an antioxidant cell protective action, cycling between its reduced (GSH) and oxidized forms (GSSG) (Ghibelli et al. 1999). Reactive oxygen species (ROS) mediated apoptotic signaling is associated with a decrease of cellular GSH levels and loss of cellular redox balance (Circu and Aw 2008) and high levels of GSH have been associated to drug resistance (Estrela et al. 2006).

As shown in Figure 7, incubation with exogenous GSH decreased the cytotoxicity induced by MTX. These results suggest that a more reduced state of cytochrome c correlates with less MTX cytotoxicity, as previously observed with TMPD or ASC.

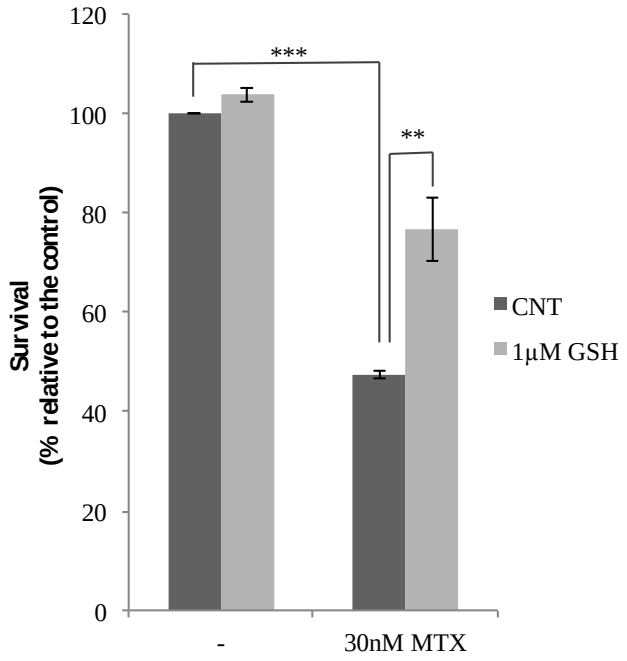


Figure 7 – Effect of GSH treatment on the cytotoxicity caused by MTX. MCF7 parental cells ($6x10^4$) were plated in 1ml medium and treated with 1µM GSH (light grey bars) for 2h before incubation with MTX. Survival was assessed by the MTT assay 4 days later. Results are expressed as % of survival compared to non treated cells and represent the mean ± SEM of at least 3 experiments. $^{**}p<0.01$, $^{***}p<0.001$.

To explore the role of GSH on MTX-dependent apoptosis we used veratridine to decrease GSH levels (Jordan et al. 2002). As shown in Figure 8, the apoptotic effect provoked by the combination of veratridine plus MTX was higher than the sumatory of both agents by themselves. Interestingly, the addition of 1μM GSH decreased MTX-induced apoptosis and counteracted the increase in apoptosis caused by veratridine.

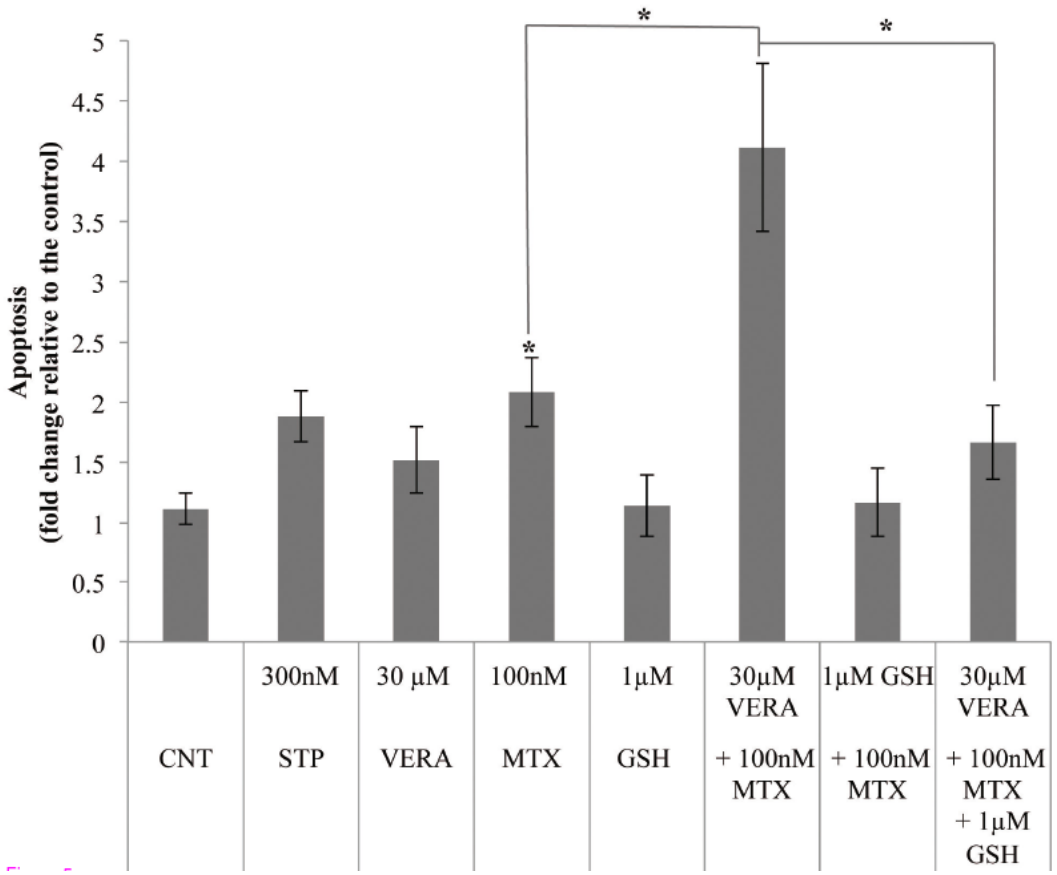


Figure 8 - Effect of veratridine (VERA) and GSH on MTX induced apoptosis. Cells were incubated with veratridine alone or in combination either with MTX or MTX plus GSH, at the indicated concentrations. Pre-treatment with veratridine was performed 6h before MTX incubation. Incubation with exogenous GSH started 8h before the addition of MTX. In the triple combination, cells were incubated 2h with GSH, then veratridine was added and 6h later treatment with MTX was performed. Apoptosis was assessed 18h after MTX addition by changes in mitochondrial membrane potential determined using the Rhodamine 123/Propidium Iodide assay and is expressed as fold change compared to untreated cells. Results represent the mean ± SE of 3 different experiments. Staurosporine (STP) was used as a positive control. * $p<0.05$.

These results indicate a possible role of GSH in MTX-induced apoptosis. It has been suggested that endogenous GSH contributes to maintain cytochrome c in its reduced state under physiological conditions and prevents its apoptotic effect (Hancock et al. 2001; Hancock et al. 2003; Vaughn and Deshmukh 2008; Ripple et al. 2010). A lower reduced environment caused by GSH depletion would favor cytochrome c induced apoptosis upon MTX incubation.

GSH endogenous levels were determined *in situ* in sensitive and resistant cells. As shown in figure 9a, GSH content was 6.2 times higher in resistant cells, indicating that the detoxifying capacity of the cytoplasm in resistant cells was higher than in sensitive cells. To analyze this, exogenous cytochrome c was incubated with either sensitive or resistant cytosolic extracts. Changes in cytochrome c redox state were measured spectrophotometrically as described. The results in Figure 9b confirmed that resistant cells had a higher capacity to reduce the exogenous cytochrome c (60%). These results support the idea that the higher reduced environment present in MTX-resistant cells would contribute to overcome the apoptotic stimuli, in this case produced by MTX, and favor the resistant phenotype.

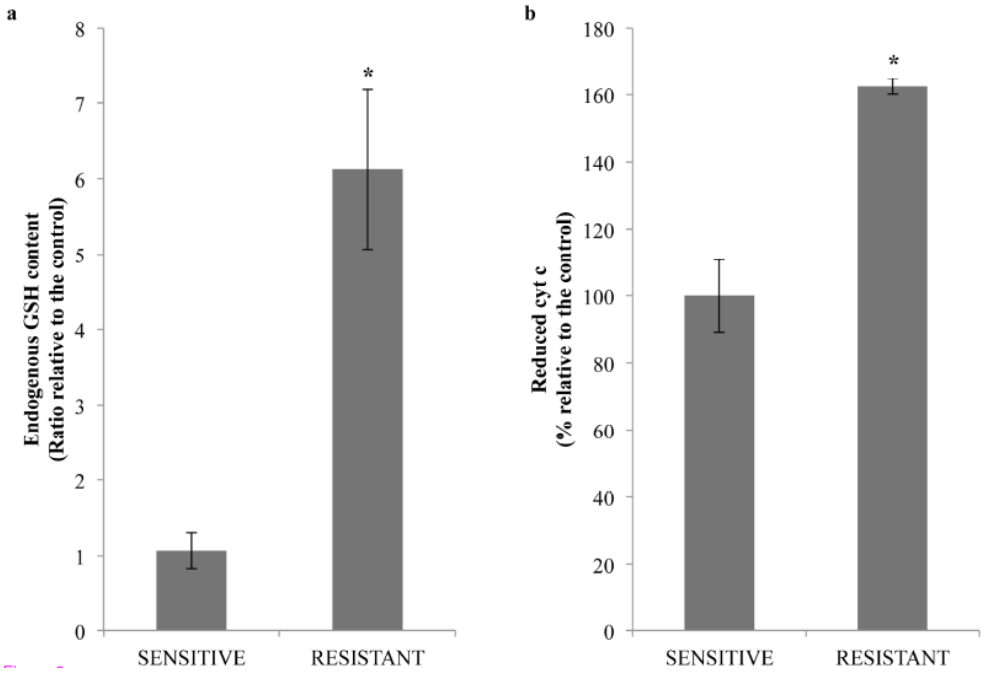


Figure 9 - Endogenous GSH levels and cytochrome c redox capacity in cytosolic extracts. (a) GSH endogenous levels were determined as described in Methods in sensitive and MTX-resistant MCF7 cells ($6x10^4$). GSH content was calculated as nmols of GSH/mg of total protein (mean ± SEM). The results are expressed as the ratio between resistant and sensitive cells. (b)**.** Cytosolic extracts from sensitive and resistant cells were obtained and incubated for 15min with 10μM of exogenous cytochrome c. A sample with DTT and no cell extract was considered as the maximum cytochrome c reduction value. Reduction level of cytochrome c was calculated as absorbance at 550 nm minus absorbance at 535 nm. The results are expressed as the percentage of reduction observed in the resistant compared to the sensitive cell extracts and represent the mean± SEM of at least three experiments. * $p<0.05$.

4.4. Endogenous levels of GST in sensitive and MTX resistant MCF7 cells

The treatment with exogenous GSH prior to MTX had the same effect on cell viability as preincubation with TMPD or ascorbate, both known cytochrome c reducing agents. Therefore, an increase in GSH would keep cytochrome c reduced and could help the cells to reduce the apoptotic effect induced by MTX. To explore more in detail this possibility and its mechanism in our model of MTX resistance we searched for genes related with GSH and the balancing redox environment of the cell.

Whole genome expression microarrays of sensitive and MTX-resistant MCF7 cells had been previously performed in the laboratory (Selga et al. 2009) and

deposited in the GEO database with series accession number GSE16648. Interestingly, analyses of the data demonstrated that different isoforms of the GST family, namely GSTM1, GSTM2 and GSTM4, were overexpressed in MCF7 resistant cells compared to their sensitive counterparts (Table 4).

Table 4 - Differentially expressed GSTs in MCF7 MTX-resistant cells. Microarray data analysis was performed with GeneSpring GX 12.0 software as described. For each GSTM isoform, it is expressed the mean of the raw value in sensitive and resistant cells, the fold change in expression after normalization of the data, as well as, the corrected *p*-value after Benjamini-Hochberg FDR filtering

Gene Symbol	Raw resistant	Raw sensitive	Fold Change	Corrected *p*-value
GSTM1	129	58	2.2	0.01288
GSTM2	182	76	2.4	0.02732
GSTM4	151	67	4.8	0.02732

Several examples in the literature have established a link between GSTM1 and GSTM4 overexpression with drug resistance (Luo et al. 2009; Hosono et al. 2010; Moyer et al. 2010; Pasello et al. 2011; Wang et al. 2011), for this reason GSTM1 and GSTM4 were selected for further study. On the other hand, GSTM2 is a muscle-specific human GSTmu isoform specially enriched in in the cytoplasm of skeletal and cardiac muscle (Abdellatif et al. 2007). Although both increased or decreased expression of *GSTM2* have been related to cancer predisposition or promotion, such as lung cancer (Tang et al. 2011) or ovarian teratoma (Han et al. 2011), no clear evidence between GSTM2 increased levels and drug resistance has been reported, and therefore this isoform was not further studied.

GSTM1 and *GSTM4* mRNA levels were validated by RT-Real Time PCR in sensitive and MTX-resistant cells. As it can be observed in Figure 10 both *GSTM1* (a) and *GSTM4* (b), were overexpressed 2.2 and 2.77-fold, respectively, in MCF7 resistant cells, confirming GSTs overexpression detected in the microarray experiments.

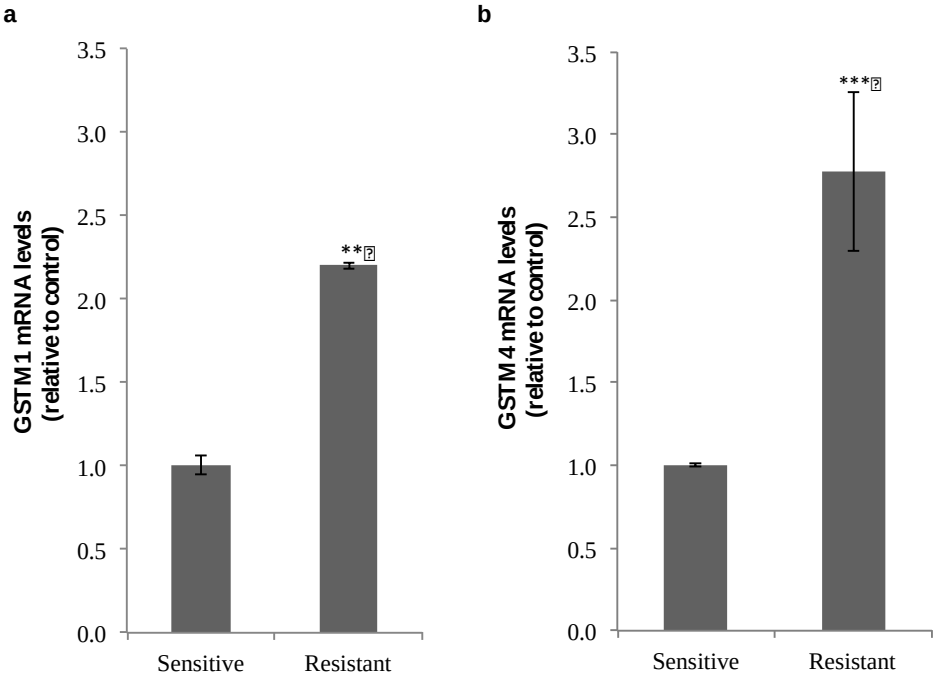


Figure 10 – Validation of *GSTM1* and *GSTM4* overexpression in MCF7 MTXresistant cells. *GSTM1* (a) and *GSTM4* (b) mRNA expression levels were determined by RT-Real Time PCR in sensitive and 10^{-6}M MTX-resistant MCF7 cells. Results are expressed as fold change in expression compared to sensitive cells and are the mean ± SE of at least 3 different experiments. ** p <0.01, *** p <0.001.
bsorbance at 535 nm. The results are expressed as the percentage of reduction observed in the resistant compared to the sensitive cell extracts and represent the mean± SEM of at least three experiments. * p<0.05.

4.5. Inhibition of *GSTM1* and *GSTM4* increases the cytotoxicity produced by MTX

To establish a role of GSTs in the sensitivity to MTX, we silenced *GSTM1* and *GSTM4* using specific template polypurine hairpins, a new class of molecules for specific and effective gene silencing developed in the laboratory (de Almagro et al. 2009; de Almagro et al. 2011).

As it can be observed in Figure 11c & d, MTX cytotoxicity increased when either GSTM1 or GSTM4 were inhibited by specific PPRH-hairpins (Figure 11a & b), demonstrating the role of these specific GSTs in diminishing the cytotoxicity produced by MTX.

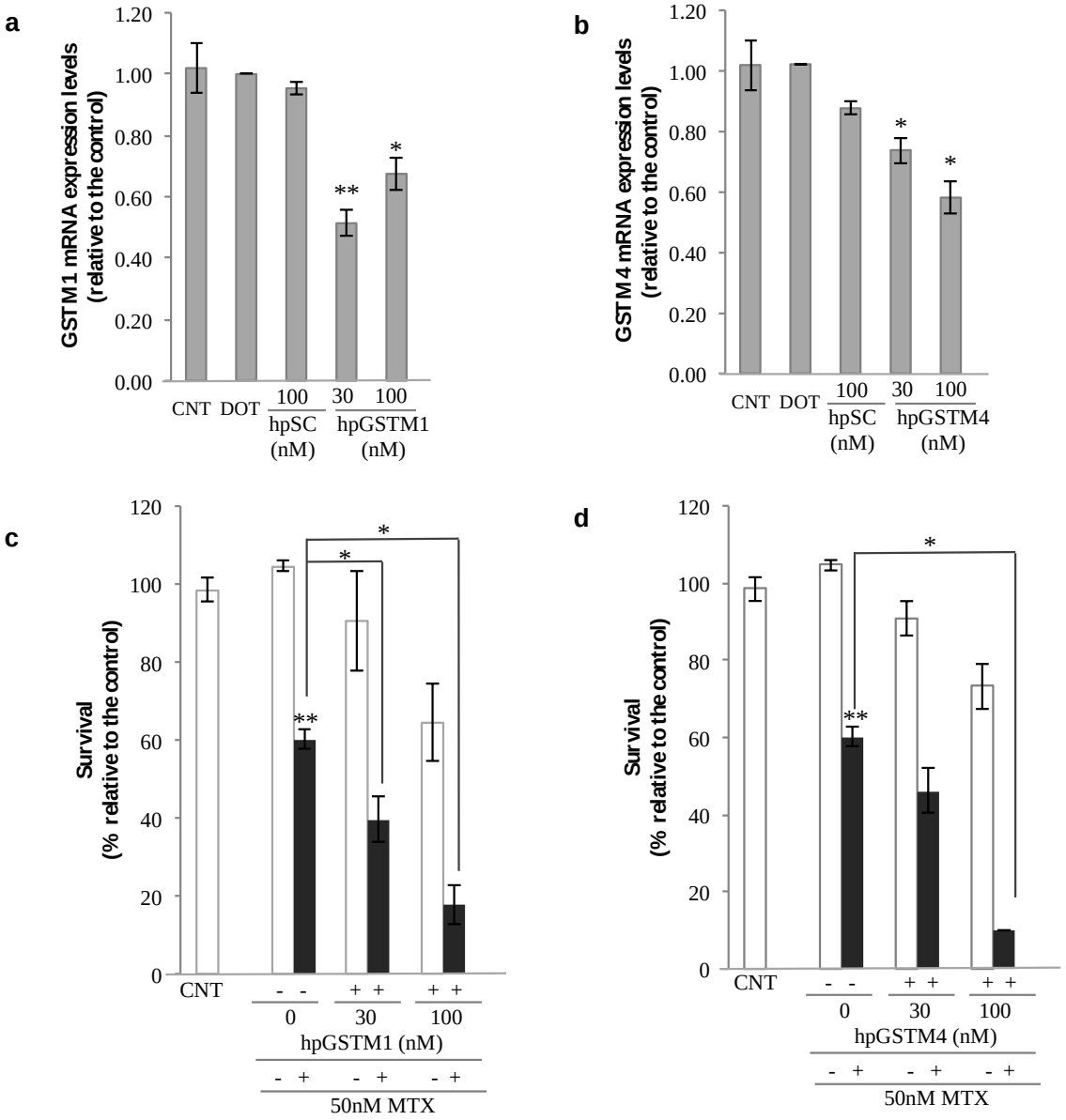


Figure 11 - Effect of GSTM1 and GSTM4 inhibition on *GSTM1* and *GSTM4* mRNA levels, and on sensitivity towards MTX. MCF7 cells ($6x10^4$) were plated in 1ml of medium and transfected 18h later with specific hairpins against GSTM1 (hpGSTM1) (a) and GSTM4 (hpGSTM4) (b). mRNA levels were determined 30h after transfection. Results are expressed as changes in expression compared to the control non-transfected cells and are the mean ± SE of at least 3 different experiments. A scrambled hairpin was used as a negative control (hpSC). Cytotoxicity upon transfection with hpGSTM1 (c) or hpGSTM4 (d) alone or in combination with MTX. MCF7 cells ($6x10^4$) were plated in 1ml of medium and transfected 18h later with either hpGSTM1 or hpGSTM4. MTX was added 24h after transfection and viability assayed by MTT 3 days later. Results are expressed as % of survival compared to the control non-transfected cells and are the mean ± SE of at least 3 different experiments. *p <0.05, **p <0.01.

GSTs, in addition to their classic catalytic functions in detoxification of electrophilic compounds, are also involved in the regulation of other mechanisms that impact cell survival pathways, such as the JNK-pathway (Townsend and Tew 2003; Tew and Townsend 2012). Interestingly, overexpression of GSTM1 (Cho et al. 2001) and GSTP (Adler et al. 1999) has been described to prevent the activation of MAPK pathway, thus avoiding the apoptosis cascade. This link with MAPK-mediated signaling could provide a possible mechanism of action of GST in drug-resistant cells. In addition, overexpression of GSTs has been associated with resistance to many therapeutic drugs (Townsend and Tew 2003), even if they are not GSTs substrates (Tew 1994; Fan and Chambers 2001). Another observation that argues in favor of GSTs playing a role in the modulation of apoptosis is the finding that Bee Venom, an inducing-apoptosis agent, increases the expression of apoptotic proteins, e.g. Bax, Bid, p53, p27, cytochrome c but decreases the expression of anti-apoptotic proteins like Bcl-2, Bcl-xL, and also GSTs (IP et al. 2008). Although there is no evidence of s-glutathionylation of cytochrome c in vivo, direct interaction between GSH and cytochrome c has been described in vitro (Deng 2006). Therefore, GST might help to maintain, directly or indirectly, cytochrome c in a reduced state.

According to our results, inhibition of GSTM1 and GSTM4 increases the sensitivity to MTX in sensitive cells, which is in keeping with the overexpression of these particular isoforms in breast cancer cells resistant to MTX. These observations suggest a role of GSTs in MTX drug resistance.

5. Conclusions

There is a relationship between cytochrome c redox state, apoptosis and development of MTX-resistance. In the presence of exogenous reducing agents of cytochrome c such as TMPD, ascorbate or GSH, cells were less prone to apoptosis, which led to a lower MTX cytotoxicity. On the other hand, depletion of endogenous GSH using veratridine caused an increase in the apoptotic action of MTX, which was reverted by the addition of exogenous GSH. Furthermore, endogenous levels of GSH were higher in MTX-resistant cells. These observations suggest that cytochrome c redox state modulates MTX sensitivity. Inhibition of GSTM1 and

GSTM4, which are overexpressed in MTX-resistant cells, caused an increase in MTX cytotoxicity. In summary, we conclude that in MCF7 breast cancer cells the overexpression of specific GSTs and increased GSH levels contribute to a more reduced environment. Thus, the presence of a more reduced cytochrome c would help the cells to avoid apoptosis and contribute to the resistant phenotype.

Acknowledgments

The work was supported by grant SAF2011- 23582 from “Plan Nacional de Investigación Científica” (Spain). Our group holds the Quality Mention from the “Generalitat de Catalunya” SGR2009-118. NM is recipient of a fellowship (APIF) from the University of Barcelona (UB). SB was recipient of an ERASMUS Placement Programme grant from University of Aveiro (UA). We thank Meryem Gonzalez and Pau Castel for their contribution to the initial phase of this work.

References

Abdellatif Y, Liu D, Gallant EM, Gage PW, Board PG, et al. (2007) The Mu class glutathione transferase is abundant in striated muscle and is an isoform-specific regulator of ryanodine receptor calcium channels. Cell Calcium 41: 429-440.

Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, et al. (1999) Regulation of JNK signaling by GSTp. EMBO J 18: 1321-1334

Alt FW, Kellems RE, Bertino JR, Schimke RT (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. J Biol Chem 253: 1357-1370.

Armstrong RN (1997) Structure, Catalytic Mechanism, and Evolution of the Glutathione Transferases. Chemical Research in Toxicology 10: 2-18.

Aupetit B, Emeric N, Toury R, Racadot O, Racadot J, et al. (1986) Stimulation of oxygen consumption at the cytochrome A3 level inhibits aldosterone biosynthesis from 18-hydroxycorticosterone. Biochim Biophys Acta 884: 270-275.

Belikova NA, Vladimirov YA, Osipov AN, Kapralov AA, Tyurin VA, et al. (2006) Peroxidase activity and structural transitions of cytochrome c bound to cardiolipin-containing membranes. Biochemistry 45: 4998-5009.

Blakley RL, Cocco L (1984) Dismutation of dihydrofolate by dihydrofolate reductase. Biochemistry 23: 2377-2383.

Brown GC, Borutaite V (2008) Regulation of apoptosis by the redox state of cytochrome c. Biochim Biophys Acta 1777: 877-881.

Borutaite V, Brown GC (2007) Mitochondrial Regulation of Caspase Activation by Cytochrome Oxidase and Tetramethylphenylenediamine via Cytosolic Cytochrome c Redox State. Journal of Biological Chemistry 282: 31124-31130.

Cho SG, Lee YH, Park HS, Ryoo K, Kang KW, et al. (2001) Glutathione S-transferase mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. J Biol Chem 276: 12749-12755.

Chu E, Grem JL, Johnston PG, Allegra CJ (1996) New Concepts for the Development and Use of Antifolates. STEM CELLS 14: 41-46.

Circu ML, Aw TY (2008) Glutathione and apoptosis. Free Radic Res 42: 689-706.

de Almagro MC, Coma S, Noe V, Ciudad CJ (2009) Polypurine hairpins directed against the template strand of DNA knock down the expression of mammalian genes. J Biol Chem 284: 11579-11589.

de Almagro MC, Selga E, Thibaut R, Porte C, Noe V, et al. (2011) UDP-glucuronosyltransferase 1A6 overexpression in breast cancer cells resistant to methotrexate. Biochem Pharmacol 81: 60-70

de Almagro MC, Mencia N, Noe V, Ciudad CJ (2011) Coding polypurine hairpins cause target-induced cell death in breast cancer cells. Hum Gene Ther 22: 451-463.

Deng H (2006) Characterization of the reaction products of cytochrome c with glutathione by mass spectrometry. Biochem Biophys Res Commun 342: 73-80.

Estrela JM, Ortega A, Obrador E (2006) Glutathione in cancer biology and therapy. Crit Rev Clin Lab Sci 43: 143-181.

Everse J, Kujundzic N (1979) Kinetics and mechanism of the reduction of horse heart ferricytochrome c by glutathione. Biochemistry 18: 2668-2673.

Fan M, Chambers TC (2001) Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. Drug Resist Updat 4: 253-267.

Ghibelli L, Coppola S, Fanelli C, Rotilio G, Civitareale P, et al. (1999) Glutathione depletion causes cytochrome c release even in the absence of cell commitment to apoptosis. FASEB J 13: 2031-2036.

Gorlick R, Goker E, Trippett T, Steinherz P, Elisseyeff Y, et al. (1997) Defective Transport Is a Common Mechanism of Acquired Methotrexate Resistance in Acute Lymphocytic Leukemia and Is Associated With Decreased Reduced Folate Carrier Expression. Blood 89: 1013-1018.

Haber D, Beverley S, Kiely M, Schimke R (1981) Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. J Biol Chem 256: 9501-9510.

Han I, Jeong SJ, Lee HJ, Koh W, Lee EO, et al. (2011) Proteomic analysis of mesenchymal stem-like cells derived from ovarian teratoma: potential role of glutathione S-transferase M2 in ovarian teratoma. Proteomics 11: 352-360.

Hancock JT, Desikan R, Neill SJ (2001) Does the redox status of cytochrome C act as a fail-safe

Hancock JT, Desikan R, Neill SJ (2003) Cytochrome c, Glutathione, and the Possible Role of Redox Potentials in Apoptosis. Ann N Y Acad Sci 1010: 446-448.

Hayes JD, Pulford DJ (1995) The Glut athione S-Transferase Supergene Family: Regulation of GST and the Contribution of the lsoenzymes to Cancer Chemoprotection and Drug Resistance Part I. Critical Reviews in Biochemistry and Molecular Biology 30: 445-520.

Hayes JD, McLellan LI (1999) Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radic Res 31: 273-300.

Hayes JD, Flanagan JU, Jowsey IR (2004) GLUTATHIONE TRANSFERASES. Annual Review of Pharmacology and Toxicology 45: 51-88.

Hosono N, Kishi S, Iho S, Urasaki Y, Yoshida A, et al. (2010) Glutathione S-transferase M1 inhibits dexamethasone-induced apoptosis in association with the suppression of Bim through dual mechanisms in a lymphoblastic leukemia cell line. Cancer Sci 101: 767-773.

Huang CC, Hsu PC, Hung YC, Liao YF, Liu CC, et al. (2005) Ornithine decarboxylase prevents methotrexate-induced apoptosis by reducing intracellular reactive oxygen species production. Apoptosis 10: 895-907.

Iverson SL, Enoksson M, Gogvadze V, Ott M, Orrenius S (2004) Cardiolipin is not required for Bax-mediated cytochrome c release from yeast mitochondria. J Biol Chem 279: 1100-1107.

IP S-W, LIAO S-S, LIN S-Y, LIN J-P, YANG J-S, et al. (2008) The Role of Mitochondria in Bee Venom-induced Apoptosis in Human Breast Cancer MCF7 Cells. In Vivo 22: 237-245.

Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA (1983) The Pharmacology and Clinical Use of Methotrexate. New England Journal of Medicine 309: 1094-1104.

Jordan J, Galindo MF, Tornero D, Benavides A, Gonzalez C, et al. (2002) Superoxide anions mediate veratridine-induced cytochrome c release and caspase activity in bovine chromaffin cells. Br J Pharmacol 137: 993-1000.

Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritov VB, et al. (2005) Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat Chem Biol 1: 223-232.

Kuznetsov AV, Schneeberger S, Seiler R, Brandacher G, Mark W, et al. (2004) Mitochondrial defects and heterogeneous cytochrome c release after cardiac cold ischemia and reperfusion. Am J Physiol Heart Circ Physiol 286: H1633-1641.

Liani E, Rothem L, Bunni MA, Smith CA, Jansen G, et al. (2003) Loss of folylpoly-gamma-glutamate synthetase activity is a dominant mechanism of resistance to polyglutamylation-dependent novel antifolates in multiple human leukemia sublines. Int J Cancer 103: 587-599.

Li M, Wang AJ, Xu JX (2008) Redox state of cytochrome c regulates cellular ROS and caspase cascade in permeablized cell model. Protein Pept Lett 15: 200-205.

Li T, Ito K, Sumi S, Fuwa T, Horie T (2009) Protective effect of aged garlic extract (AGE) on the apoptosis of intestinal epithelial cells caused by methotrexate. Cancer Chemother Pharmacol 63: 873-880.

Luo W, Gangwal K, Sankar S, Boucher KM, Thomas D, et al. (2009) GSTM4 is a microsatellite-containing EWS/FLI target involved in Ewing's sarcoma oncogenesis and therapeutic resistance. Oncogene 28: 4126-4132.

McLellan LI, Wolf CR (1999) Glutathione and glutathione-dependent enzymes in cancer drug resistance. Drug Resist Updat 2: 153-164.

Mencia N, Selga E, Rico I, de Almagro MC, Villalobos X, et al. (2010) Overexpression of S100A4 in human cancer cell lines resistant to methotrexate. BMC Cancer 10: 250.

Mencia N, Selga E, Noe V, Ciudad CJ (2011) Underexpression of miR-224 in methotrexate resistant human colon cancer cells. Biochem Pharmacol.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.

Moyer AM, Sun Z, Batzler AJ, Li L, Schaid DJ, et al. (2010) Glutathione pathway genetic polymorphisms and lung cancer survival after platinum-based chemotherapy. Cancer Epidemiol Biomarkers Prev 19: 811-821.

Nishimura G, Proske RJ, Doyama H, Higuchi M (2001) Regulation of apoptosis by respiration: cytochrome c release by respiratory substrates. FEBS Lett 505: 399-404.5.

Noe V, Alemany C, Ciudad CJ (1995) Determination of dihydrofolate reductase gene amplification from single cell colonies by quantitative polymerase chain reaction. Anal Biochem 224: 600-603.

Ott M, Zhivotovsky B, Orrenius S (2007) Role of cardiolipin in cytochrome c release from mitochondria. Cell Death Differ 14: 1243-1247.

Pasello M, Manara MC, Michelacci F, Fanelli M, Hattinger CM, et al. (2011) Targeting glutathione-S transferase enzymes in musculoskeletal sarcomas: a promising therapeutic strategy. Anal Cell Pathol (Amst) 34: 131-145.

Ripple MO, Abajian M, Springett R (2010) Cytochrome c is rapidly reduced in the cytosol after mitochondrial outer membrane permeabilization. Apoptosis 15: 563-573.

Sarti P, Antonini G, Malatesta F, D'Itri E, Brunori M, et al. (1992) Spectral analysis of cytochromes in rat heart myocytes: transient and steady-state photodiode array spectrophotometry

Sarti P, Antonini G, Malatesta F, Brunori M (1992) Respiratory control in cytochrome oxidase vesicles is correlated with the rate of internal electron transfer. Biochem J 284 (Pt 1): 123-127.measurements. Arch Biochem Biophys 299: 8-14.

Sirotnak FM, Moccio DM, Kelleher LE, Goutas LJ (1981) Relative Frequency and Kinetic Properties of Transport-defective Phenotypes among Methotrexate-resistant L1210 Clonal Cell Lines Derived in Vivo. Cancer Research 41: 4447-4452.

Selga E, Noe V, Ciudad CJ (2008) Transcriptional regulation of aldo-keto reductase 1C1 in HT29 human colon cancer cells resistant to methotrexate: role in the cell cycle and apoptosis. Biochem Pharmacol 75: 414-426.

Selga E, Morales C, Noe V, Peinado MA, Ciudad CJ (2008) Role of caveolin 1, E-cadherin, Enolase 2 and PKCalpha on resistance to methotrexate in human HT29 colon cancer cells. BMC Med Genomics 1: 35.

Selga E, Oleaga C, Ramirez S, de Almagro MC, Noe V, et al. (2009) Networking of differentially expressed genes in human cancer cells resistant to methotrexate. Genome Med 1: 83.

Silva AM, Oliveira PJ (2012) Evaluation of respiration with clark type electrode in isolated mitochondria and permeabilized animal cells. Methods Mol Biol 810: 7-24.

Suto D, Sato K, Ohba Y, Yoshimura T, Fujii J (2005) Suppression of the pro-apoptotic function of cytochrome c by singlet oxygen via a haem redox state-independent mechanism. Biochem J 392: 399-406.

Tang SC, Wu MF, Wong RH, Liu YF, Tang LC, et al. (2011) Epigenetic mechanisms for silencing glutathione S-transferase m2 expression by hypermethylated specificity protein 1 binding in lung cancer. Cancer 117: 3209-3221.

Tew KD (1994) Glutathione-associated enzymes in anticancer drug resistance. Cancer Res 54: 4313-4320.

Tew KD, Townsend DM (2012) Glutathione-S-transferases as determinants of cell survival and death. Antioxid Redox Signal.

Townsend DM, Tew KD (2003) The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene 22: 7369-7375.

Vaughn AE, Deshmukh M (2008) Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. Nat Cell Biol 10: 1477-1483.

Wang CH, Wu HT, Cheng HM, Yen TJ, Lu IH, et al. (2011) Inhibition of glutathione S-transferase M1 by new gabosine analogues is essential for overcoming cisplatin resistance in lung cancer cells. J Med Chem 54: 8574-8581.

Chapter III - General Conclusion

Methotrexate is a DNA-damaging agent widely used in chemotherapy to treat a variety of cancers, triggering p53-dependent apoptotic intrinsic pathway. However, the occurrence of resistance limits its effectiveness.

Cytochrome c, as a key component of apoptotic intrinsic pathway, is less capable of inducing apoptotic cascade when is in its reduced state. Thus, in the present work MCF7 breast cancer cells were used as a model to set up a study of the relationship among redox state of cytochrome c, apoptosis and the development of resistance to methotrexate.

The redox state of cytochrome c is mainly regulated by the intracellular redox environment in which GSH and GST have an important role. In the presence of exogenous reducers of cytochrome c (TMPD, ascorbate, GSH), methotrexate-treated cells showed less mortality and apoptosis. On the other hand, depletion of endogenous GSH increased the apoptotic action of methotrexate, which was reverted by the addition of exogenous GSH. Furthermore, resistant cells to methotrexate showed higher endogenous levels of GSH. MTX-resistant cells displayed an overexpression of specific GSTMs, GSTM1 and GSTM4, and when inhibited an increase in methotrexate cytotoxicity in sensitive cells arose.

According to the results, overexpression of specific GSTs and increased endogenous GSH levels appear to help to keep the cytochrome c more reduced. This seems to help the cells to avoid the apoptotic effect of MTX leading to the development of resistance in human cancer cells.

In conclusion, we can establish a relationship between cytochrome c redox state, apoptosis and development of MTX-resistance in MCF7 breast cancer cells.

Annex – Experimental Protocols

Annex I

MTT assay protocol (Mosmann, 1983)

Cell viability was assessed by the MTT assay as described by Mosmann (1983). MTT assay is based on the ability of viable cells to reduce a tetrazolium salt (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide-MTT) to an intercellular purple formazan which can be solubilized and quantified spectrophotometrically at the wavelength 570nm. The signal produced is linear to the quantity of viable cells.

Solutions:

MTT solution 0.25%

Sodium succinate 270µM

Solubilization reagent: 10% SDS in DMSO and 0.57% acetic acid

Method:

1st Day

Seed $6x10^4$ cells in a 6 well-plate in 1ml of –GHT medium and leave for an overnight period to attach

2nd Day

Treat the cells with MTX alone or in combination with other compounds:

Compound	*Pre-incubation period*	*MTX 30nM incubation period (alone or in combination)*
5µM TMPD	6 hours	3 days
300µM Ascorbate	6 hours	6 days
1µM GSH	2 hours	3 days

XX Day

1. Assess the survival after the MTX incubation period:
2. Without removing the medium, add 200µl of MTT 0.25% and 700µl of 270µM sodium succinate solution
3. Incubate for 2h at 37ºC
4. After this 2h, remove the MTT + sodium succinate solution and add 1ml of the solubilization reagent
5. Measure the absorbance at 570nm
6. Express the results as percentage of cell survival relative to control (untreated cells)

Annex II

Rhodamine 123/IP protocol

A common feature of the early stages of apoptosis is the disruption of active mitochondria. Alterations in the mitochondrial membrane are the main event of the mitochondrial disruption; this could be due to the formation of transient pores, allowing the passage of ions and small molecules. The mitochondrial transient permeabilization leads to decoupling of the respiratory chain and cytochrome c release into the cytosol. Rhodamine 123 is a cell permeant cationic fluorescent dye that is readily taken up by active mitochondria without causing cytotoxicity, allowing to identify earlier apoptotic cells, which are not capable of keeping the rhodamine 123 inside the mitochondria. In the later stages of apoptosis or in necrotic stage, the plasma membrane is disrupted and the cell becomes permeable. This permits to label the later apoptotic cells, since the propidium iodide can enter into the cell to selectively stain the DNA.

Solutions:

PBS 1X:
PBS 1X + 1% Bovine Serum Albumin (BSA)

Fluorochromes:

Rhodamine 123 1µg/µl – fluorescent emission at 525nm
Propidium Iodide 5µg/µl – fluorescent emission at 630nm

Method

1st Day

Seed 6x10^4 cells in a 6 well-plate in 1ml of –GHT medium and leave for an overnight period to attach

2nd Day

Treat the cells with MTX alone or in combination with other compounds:

Compound	*Pre-incubation period*	*MTX 50nM;100nM incubation period (alone or in combination)*
5µM TMPD	12 hours	18 hours
1mM Ascorbate	12 hours	18 hours
300nM Staurosporine	18 hours	XXX
Veratridine 30µM	6 hours	18 hours
1µM GSH	8 hours	18 hours
1µM GSH + 30µM Veratridine	2 hours + 6 hours	18 hours

Day XX

1. Without removing the medium, add 5µl of Rhodamine 123 and incubate the cells for 30 minutes at 37ºC
2. Collect the medium and centrifuge at 3500rpm at 4ºC; discard the supernatant (SN) – to collect the de-attached cells (dead cells)
3. Harvest the adherent cells by trypsinization. Centrifuge at 3500 rpm at 4ºC. Discard the SN.
4. Wash with 1ml PBS 1X + 1% BSA. Centrifuge at 3500 rpm at 4ºC. Discard the SN.
5. Ressuspend the pellet with 500µl PBS 1X + 1% BSA + 0.5µl IP
6. Analyze the samples by flow cytometry

Annex III

Preparation of cytosolic extracts

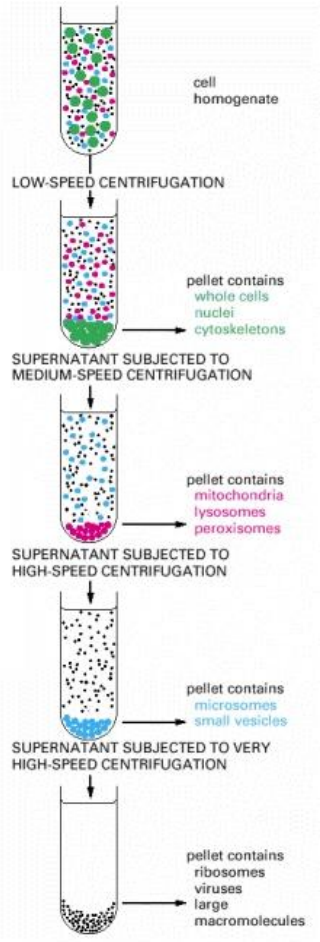
Solutions

Non-denaturant cell lysis buffer: sucrose 250mM, KCl 10mM, HEPES pH 7.4 20mM, $MgCl_2$ 1.5mM, EDTA 0.5mM, EGTA 0.5mM, ultrapure H_2O; add immediately before use protease cocktail inhibitors 1:100 and PMSF 100mM for a final concentration of 1mM

PBS 1X:

From a 70% confluent 100mm dish:

1. Remove the medium from the cells and wash the cells twice with 2 ml of PBS
2. Remove PBS and add 2ml of PBS to detach the cells using a scraper and transfer the cell suspension to a 10ml falcon tube.
3. Wash the plate once again with 3ml PBS and scrape the dish to detach the remaining cells
4. Centrifuge cells at 3500rpm at 4ºC for 10 min
5. Discard the SN and resuspend cells in 4ml of cold lysis buffer
6. Vórtex 10s, incubate 5min on ice, vórtex10s again
7. Homogenize the cells using a Teflon pestle operated at 730rpm; stroke the cell suspension placed in a glass potter 20times.
8. Quantify the total protein by Bradford assay
9. Use the homogenates immediately to prepare the cytosolic extracts
10. Centrifuge at 3500rpm for 10 min at 4ºC 2 volumes of the homogenate prepared before, to pull down the whole cells, nuclei and cytoskeleton



11. Collect the SN (cytosolic proteins and organelles) and centrifuge it at 15000rpm for 30min at 4ºC to pull down the organelles
12. Collect the SN (cytosolic fluid)

Measurement of cytochrome c redox state upon incubation with cytosolic extracts

Solutions:

Cytochrome c 100µM

Cytochrome c 10µM

Ascorbate 100mM

DTT 100mM

Method:

1. Incubate the cytosolic extract with 10µl cytochrome c 100µM for 15min at 37ºC
2. Measure the absorbance spectra between wavelengths of 535-550nm
3. **Control for reduction:** incubate 10mM of Ascorbate or DTT with cytochrome c 10µM

Annex IV

Determination of GSH endogenous levels in sensitive and MTX-resistant cells

The following procedure allows the measuring of GSH *in-situ* as a cell based assay using the Glutathione Assay Kit, Fluorimetric (Sigma-Aldrich®).

Solutions:

Assay buffer

Monochlorobimane Solution (substrate)

Lysis buffer 1X

Glutathione S-Transferase (40U)

Reduced Glutathione 1mM

Method:

1st Day

Seed 6x10^4 cells in a 96 well black fluorimeter plate with transparent in 100µl of –GHT medium and leave for an overnight period to adhere

2nd Day

1. Remove the medium and wash the cells twice with 200µl PBS 1X
2. Add 97.5µl of Assay Buffer and 2.5µl of monochlorobimane to each sample well and incubate at room temperature in the fluorimeter
3. At the same time, prepare the standard curve in the same plate as following:

GSH Standard	*1mM Reduced Glutathione (µl)*	*Assay Buffer (µl)*	*GST Enzyme (µl)*	*Monochlorobimane Solution (µl)*
Blank	-	92.5	5	2.5
1.3nmoles	1.3	91	5	2.5
2.5nmoles	2.5	90	5	2.5
5nmoles	5	87.5	5	2.5
10nmoles	10	82.5	5	2.5

4. Measure the fluorescence at 20minutes intervals for 1h

The optimal wavelengths for measuring the fluorescence of the GSH-Monochlorobimane adduct are:

$\Lambda_{excitation}$ = 390nm

$\Lambda_{emission}$ = 478nm

Annex V

RT-Real-Time PCR Protocol

Reagents:

RNA Extraction

Ultraspec ™ RNA Reagent

Chlorophorm

Ethanol

Isopropanol

Ethanol 70% Free RNAse

DEPC H_2O Free RNAse

RT Mix (V_f = 20µl)

Buffer 1X: 50mM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$

DTT 10mM

Random Hexamers 125ng/µl

dNTPs 500µM each

RNAse inhibitor 20U

MLV-RT 200U

RNA 1µg

Master Mix (V_f = 20µl)

Master Mix Buffer 2X

SYBR Green 1/1000

Primer Forward 1µg/µl

Primer Reverse 1µg/µl

3µl cDNA product dilution 1/10

DEPC H_2O

Method:

RNA Purification	From a 70% confluente dish: • Extraction using Ultraspec™ RNA Reagent and RNAse-free chlorophorm • Precipitation with isopropanol • Wash with ethanol 70% • Quantification using Nanodrop 1000 Spectrophotometer
Reverse Transcription Reaction	• Incubation of the Reaction Mix (RT) at 37ºC for 1h
PCR Reaction	• Mix the all the reagents of the Master Mix and the specific primers for each gene • PCR reaction (40 cycles) using StepOnePlus™ detection system: - Denaturation 94ºC 1min + 30sec - Primer Annealing 59ºC 1min - Primer Extension 72ºC 1min - Final Extension 72ºC 7min
Gene expression levels quantification	• Quantitative ΔΔCt method • Normalization against HPRT each sample

Annex VI

Silencing Gene Expression using template PPRHs (de Almagro, 2009)

Brief introduction

Suppression of gene expression constitutes a powerful therapeutical tool to inhibit the synthesis of proteins involved in pathological processes. It have been developed several types of molecules to decrease gene expression such as mainly antisense oligonucleotides (aODNs), small interfering RNAs (siRNAs) and triplex-forming oligonucleotides (TFOs). However, each of these molecules reveals disadvantages such as lack of stability or specificity. To overcome these issues hairpins, like Polypurine reverse-Hoogsteen hairpins (PPRHs), and other molecules were developed (Ryan, 1998; Sorensen, 2004; Brunet, 2005).

Hairpins consist of two DNA strands linked by a loop (Giovannangeli, 1991-, Kandimalla, 1995; Aviñó, 2001 and 2002), where the DNA strands are linked by Watson-Crick (WC) bonds. Hoogsteen bonds are hydrogen bonds between nucleic acids, with a different code than WC bonds and this kind of linkage allows the formation of triplex structure. This triplex DNA structure is formed when a third DNA strand binds to the major groove of double helix via Hoogsteen hydrogen bonding (Felsenfeld & Rich, 1957). According to the Hoogsteen geometries, the hairpins can be either parallels or antiparallels; antiparallel hairpins bind to polypyrimidine stretches in the DNA target sequence, whereas parallel hairpins bind to polypurine stretches. The intramolecular linkage of the hairpin is formed by reverse hoogsteen bonds, and union with the target sequence is mediated by Watson-Crick, d(G#G.C) and d(A#A.T). Accordingly, PPRHs are formed by two antiparallel homopurine domains link by a five-thymidine loop (Aviñó, 2002; Coma, 2005) and one of the homopurine strands binds with antiparallel orientation (by Watson-Crick) to the polypyrimidine target sequence forming a triplex. The polypyrimidine stretches, the target of PPRHs are mostly found in introns, promoters and with less probability in exons and almost all genes contain this type of sequences, implying that PPRHs could be used to target a great number

of genes (Almagro, 2009). For this reason, PPRHs may represent a new tool to inhibit gene expression in cancer therapy.

Advantages of using PPRHs

- More cytotoxic than aODNs, siRNAs and PPRH structures lacking Hoogsteen bonds
- More stable compared with modified aODNs, without the need for modified nucleotides
- Easy to synthesize and inexpensive, as they are very stable non-modified single stranded DNA oligonucleotides, with no special handling requirements in contrast to siRNAs

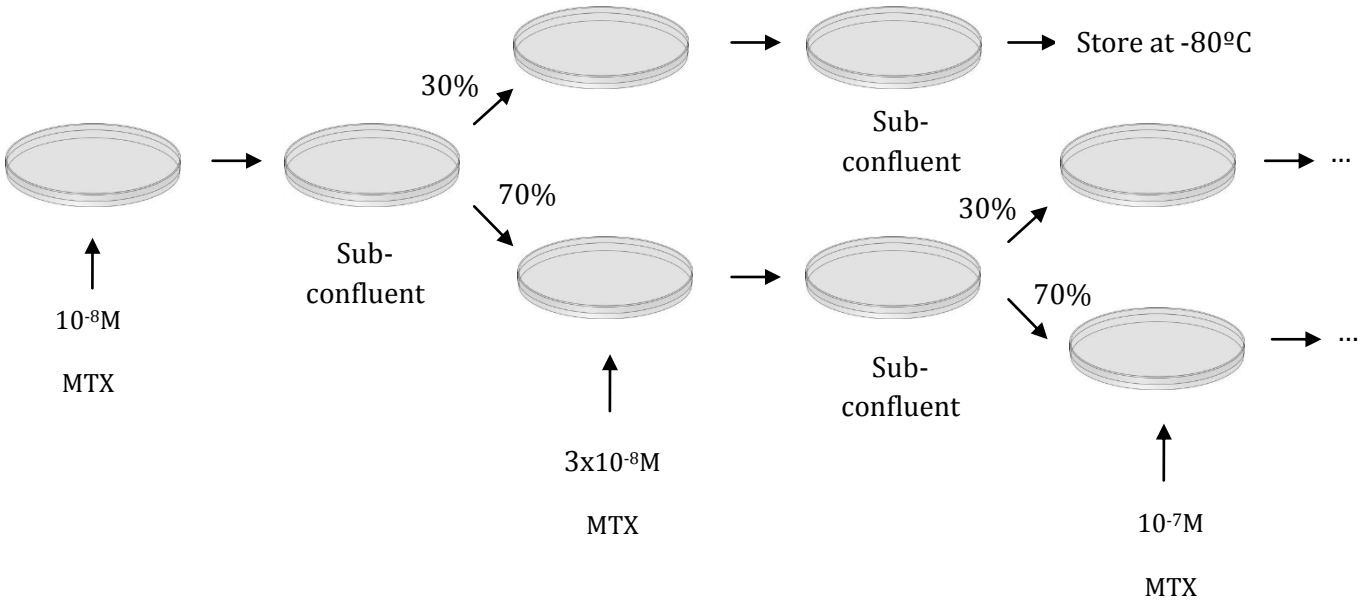
Method

Design of PPRHs	• Search for the the polypyrimidine stretches in the target sequence to design the specific hairpins using: www.spi.manderson.org/tfo (Anderson Cancer Center) • BLAST analysis
Cell transfection	• Seed $6x10^4$ cells in 6-well plate in 1ml of –GHT the night before transfection • Mix each hairpin with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) at appropriate oligonucleotide-DOTAP molar ratio 1:100 for 15min at room temperature before lipofecting the cells
Monitoring specificity	• **Cytotoxicity:** 1. After 24h, treat the transfected cells with MTX 30 and 100nM 2. Assess the viability by MTT assay 3 days later, as described in Annex I • **mRNA levels:** 1. Leave the transfected cells for 30h before RNA extraction and gene expression quantification. 2. Determine gene expression as described in Annex V 3. Include a scrambled hairpin as a negative control and an unrelated gene

Annex VII

Development of resistant cell lines MCF7-R Protocol (Selga, 2008)

Treat a semi-confluent 100mm plate of sensitive MCF7 cells with MTX 10^{-8}M. The cells that survive are left to growing until getting sub-confluent. 70% of these cells are treated with $3x10^{-8}$M and 30% are maintained at 10^{-8}M until getting enough cells to freeze or keep at -80ºC. This procedure is repeated in every increment of stepwise concentrations in factors of three until reach to 10^{-6}M of MTX, as represented in the scheme below:



Annex VIII

Oxygen consumption using the Clark type oxygen electrode protocol

The Hansatech oxygen electrod disc is a Clark type oxygen electrode, also known as a polarographic sensor. It is an electrochemical oxygen analyzer based on electrochemical reduction of O_2 at a negatively charged electrode. This type of electrode consists of a silver anode and platinum cathode in contact with an electrolyte solution, normally a 50% saturated solution of KCl. The electrode section is separated by a semi-permeable membrane, normally Teflon® membrane, which is permeable to gases but not reducible ions. Oxygen can pass the membrane and reacts with the platinum cathode, becoming reduced. Applying a polarizing voltage of 700mV ionizes the electrolyte and initiates current flow. The anode provides electrons for the cathode reaction, becoming oxidized.

Anode: $Ag \rightarrow Ag^+ + e^- \rightarrow Ag^+ + Cl^- \rightarrow AgCl$

Cathode: $O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + OH \rightarrow H_2O_2 + 2e^- \rightarrow 2OH^-$

The reduction of oxygen allows a current to flow creating a potential difference which is recorded by computer software. The current flowing is proportional to the oxygen concentration giving a signal, thus the trace corresponds to oxygen consuming. The solution containing the cells is stirred constantly in a closed chamber connected to a water bath at 37ºC.

Method

1. Seed $6x10^4$ cells in a 6 well-plate in 1ml of –GHT medium and leave for an overnight period to adhere
2. Harvest the cells by trypsinization
3. Centrifuge at 3500 rpm at 4ºC and resuspend the pellet in 1ml pre-warmed at 37ºC PBS 1X
4. Before start to record the oxygen consumption, pre-warm the bath Record the oxygen consumption in absence or presence of TMPD 5µM
5. Determine the steeper slope, representing the oxygen consumption rate