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PLATELETS DECREASE CHEMOTHERAPY-INDUCED CANCER CELL DAMAGE BY INCREASING CELL SURVIVAL: MECHANISMS AND SIGNIFICANCE

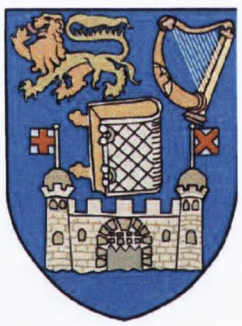
by

Aneta Radziwon

being a thesis submitted for the degree of Doctor of Philosophy (Pharmacology)

at

TRINITY COLLEGE DUBLIN



Under the supervision and direction of

Professor Marek W. Radomski

SCHOOL OF PHARMACY AND PHARMACEUTICAL SCIENCES
TRINITY COLLEGE DUBLIN

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DECLARATION

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SUMMARY

Cancer cells grow without the restraints of feedback control mechanisms that regulate normal tissue or organ growth, such as apoptosis, leading to increased cancer cell survival. Deregulation of apoptosis has been implicated in carcinogenesis. Platelets play an important role in various stages of cancer progression such as angiogenesis, invasion and metastasis (Jurasz, Alonso-Escolano et al. 2004). Cancer cell survival was affected by platelets. The general objective of my PhD research was to study the role of platelets in chemotherapy-induced cancer cell death and survival. Therefore, the studies focused on the effects of platelets on apoptosis, cell cycle regulation, DNA damage repair, mitogen-activated protein kinase pathways and telomerase activity.

Human colonic adenocarcinoma Caco-2 and human ovarian adenocarcinoma 59M cells were incubated with 5-fluorouracil (1 μg/ml – 3000 μg/ml) or paclitaxel (1 μg/ml - 200 μg/ml) in the presence or absence of platelets (1.5 x 10^8/ml) for 1, 24 or 72 hrs. Following incubation, cancer cells were harvested and cell survival/death assayed using flow cytometry, Western blotting, ELISA, real-time PCR, microscopy and TaqMan® Gene Expression Assays. Finally, proteomics was used to study the release of factors during platelet-cancer cell interactions. The results of the studies show that platelets and their releasate: (1) have the ability to modulate chemotherapeutic agent-induced cancer cell apoptosis; (2) upregulate the anti-apoptotic genes and downregulate the pro-apoptotic ones; (3) increase the number of cancer cells in the synthesis of DNA (S) and mitosis (G_2/M) phases; (4) decrease the number of cancer cells in the quiescent (G_0/G_1)

phase; (5) upregulate the expression of cyclins A, B1, D1 and E; (6) upregulate DNA repair proteins such as BRCA1, Chk1, Mre11 and p95/Nbs1; and (7) upregulate MAPK pathways such as ERK 1, 2; p38 and JNK.

It is concluded that platelets have the ability to decrease cancer cell death and increase their survival, which may contribute to cancer cell resistance to anti-cancer chemotherapy.

LIST OF ABBREVIATIONS

A1	Bcl-2-related protein A1
ACTB	Beta-actin
ADP	Adenosine diphospate
Apaf-1	Apoptotic protease-activating factor-1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist/killer
BCAP31	B-cell receptor-associated protein 31
BCL3	B-cell lymphoma 3-encoded protein
BCL2A1	Bcl-2-related protein A1
BCL2L11	Bcl-2-like protein 11
BCL10	B-cell lymphoma/leukemia 10
BCL-W	Bcl-2-like protein 2
BCL-X	Bcl-2-associated X protein or Bax
BER	Base excision repair
Bid	pro-apoptotic Bcl-2 protein
BIK	Bcl-2-interacting killer
BIRC5	Survivin; baculoviral inhibitor of apoptosis repeat-containing 5
BNIP3L	Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3-like
BOK	Bcl-2-related ovarian killer protein
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein

CASP2	Caspase 2
CASP3	Caspase 3
CASP6	Caspase 6
CASP8AP2	Caspase 8-associated protein 2
CD39	Ectonucleoside triphosphate diphosphohydrolase 1
CD41	Platelet-endothelium attachment receptor, Platelet Glycoprotein IIb
CD61	Platelet-endothelium attachment receptor, integrin beta-3
CD62	Platelet-endothelium attachment receptor, P-selectin
CD95	Tumor necrosis factor receptor superfamily member 6
Cdks	Cyclin dependent kinases
CFLAR	CASP8 and FADD-like apoptosis regulator
Chk1	Checkpoint 1
Chk2	Checkpoint 2
CRADD	Death domain-containing protein CRADD
DAPK1	Death-associated protein kinase 1
DKC1	Dyskerin
DNA-PK	DNA-dependent protein kinase
DSBs	Double strand breaks
E2F	Transcription factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK 1, 2	Signal-regulated protein kinase
ERK5	Extracellular regulated kinase 5
FGF	Fibroblast growth factor

5FU	5-Fluorouracil
G_1 phase	Gap between mitosis and S phase
G_2 phase	Gap between S phase and meiosis
G_0 phase	Quiescent or arrested phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPIb	Glycoprotein Ib
GPIIb/IIIa	Glycoprotein IIb/IIIa
GPIa/IIa	Glycoprotein Ia/IIa
HCC	Hepatocellular carcinoma
HR	Homologous recombination
Hsp70	Heat shock 70 kDa protein
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA
γ-H2A.X	Histone H2A.X phosphorylated on serine-139
IKBKE	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IKBKG	NEMO, inhibitor of nuclear factor kappa-B kinase subunit gamma
IL-6	Interleukin 6
JNK	c-jun N-terminal kinase
Ku70/Ku80	Complex, DNA double stranded break repair
LPS	Lipopolysaccharide
LRDD	Leucine-rich repeats and death domain containing
LTB	Lymphotoxin-β
MAPK	Mitogen-activated protein kinase
Mcl-1	Induced myeloid leukemia cell differentiation protein Mcl-1

NER	Nucleotide excision repair
NFκB1	Nuclear factor NF-kappa-B p105 subunit
NFκB2	Nuclear factor NF-kappa-B p100 subunit
NFκBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NFκBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
NFκBIZ	NF-kappa-B inhibitor zeta
NHEJ	Non-homologous end joining
MeCN	Acetonitrile
MMP	Matrix metalloproteinase
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
M phase	Phase of mitosis
MMR	Mismatch repair
MRN	Mammalian complex of Mre11/Rad50/Nbs1
NALP1	Implicated in cell responses to apoptotic and inflammatory stimuli
NO	Nitric oxide
NOS	Nitric oxide synthase
p21 protein	Cyclin-dependent kinase inhibitor 1
p53	Tumour suppressor gene
p73	Tumour suppressor protein
PARP-1	Poly (ADP-ribose) polymerase 1
PARP	Poly (ADP-ribose) polymerase

PDGF	Platelet-derived growth factor
PF-4	Platelet factor 4
PGI_2	Prostacyclin
PI	Propidium Iodide
PMAIP1	Noxa, phorbol-12-myristate-13-acetate-induced protein 1
PMP	Platelet-derived microparticles
PLT	Platelets
PTX	Paclitaxel
PYCARD	Apoptosis-associated speck-like protein containing a CARD
Rad52 gene	Protein Rad encoded
RAP1	Ras-proximate-1
REL	C-Rel proto-oncogene protein
RIPK2	Receptor-interacting serine/threonine-protein kinase 2
Rb	Protein retinoblastoma
RPA	Replication protein A
ROS	Reactive oxygen species
S phase	Phase of DNA synthesis
SDSA	Synthesis-dependent strand annealing
SMC	Smooth muscle cell
SSBs	Single strand breaks
TCIPA	Tumour cell-induced platelet aggregation
TEAB	Triethylammonium bicarbonate buffer
TGF- β	Tansforming growth factor-β
TGY	Threonine-glycine-tyrosine
TILDA	TaqMan® Gene Expression Assays

TNF	Tumour necrosis factor
TNF1A	Tumor necrosis factor-α
TNFSF10	TNF-related apoptosis-inducing ligand (TRAIL)
TRAP	Telomeric Repeat Amplification Protocol
TRF	Terminal Restriction Fragment (method of measuring the length of telomere)
TXA_2	Thromboxane A_2
WRN	Werner syndrome protein
VSMCs	Vascular smooth muscle cells
VTE	Venous thromboembolism
vWF	von Willebrand factor
XIAP	X-linked Inhibitor of Apoptosis Protein

INTRODUCTION AND BACKGROUND

Cancer and platelets

In 1865, the French physician Armand Trousseau reported a high incidence of venous thrombosis in patients with gastric carcinomas, and the migratory venous thrombosis due to cancer was named Trousseau's syndrome. Later on, in 1878, Theodor Billroth showed on autopsy that human tumour cells are frequently found in association with thrombi. Recent clinical and experimental data confirm the relationship between cancer cells and blood platelets. For instance, thrombocytosis (increased platelet numbers) is often detected in cancer patients. In addition, thrombocytosis is a poor prognostic factor in stomach, pancreas, liver, ovary, breast, kidney, colon, lung and prostate cancer (Tanaka 1981; Pasquini 1995; Santos, Rodrigues et al. 2001).

Recent experimental evidence has shown that platelets contribute to different stages of cancer progression such as angiogenesis, invasion, intravasation, survival in circulation, extravasation and finally metastasis (Gupta and Massagué 2004; Jurasz, Alonso-Escolano et al. 2004).

Angiogenesis is essential for tumour development, because this process activates the growth of new capillaries, which supply cancer with nutrition and oxygen (Folkman 1995). Human platelets contribute to cancer-induced angiogenesis by releasing vascular endothelial growth factor (VEGF) (Salven, Orpana et al. 1999). Malignant tumour cells have the ability to aggregate platelets (Radomski, Jenkins et al. 1991). Cancer cells induce platelet aggregation and this process is often referred to as tumour cell-induced platelet aggregation (TCIPA). Adhesion receptors are required to support TCIPA. These include integrin platelet receptors such as GPIIb/IIIa, the members of leucine-

rich family of receptors (GPIb/IX/V) and selectins (P-selectin) (Larsen, Celi et al. 1989; Radomski 1993; Oleksowicz, Mrowiec et al. 1995; Oleksowicz 1997). In addition, platelets upon activation release from α-granules and dense bodies a variety of angiogenesis-regulating factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF- β) (Amirkhosravi 1999), which can be used by tumour cells for growth (Honn 1992; Janowska-Wieczorek, Wysoczynski et al. 2005). In addition, platelets are responsible for facilitating the adhesion of tumour cells to the endothelium tissue (Mehta 1984). Some studies indicated that platelets coat cancer cells to avoid immune system response and protect tumour cells from high shear forces in flowing blood, thus increasing their **survival** in circulation (Jurasz, Alonso-Escolano et al. 2004).

Platelets also promote cancer cell invasion to disease-free tissues and organs. In order to invade, tumour cells have the ability to degrade and remodel the extracellular matrix via release of various proteolytic enzymes. These include matrix metalloproteinases (MMPs), zinc-dependent endopeptidases, which break down extracellular matrix proteins (Sternlicht and Werb 2001). MMP-2 and MMP-9 have been implicated in cancer invasion (Jurasz, Chung et al. 2002; Jurasz, Alonso-Escolano et al. 2004). Our research group has recently reported that platelets stimulate invasiveness of tumour cells via increased expression of MMP-9 (Alonso-Escolano, Medina et al. 2006).

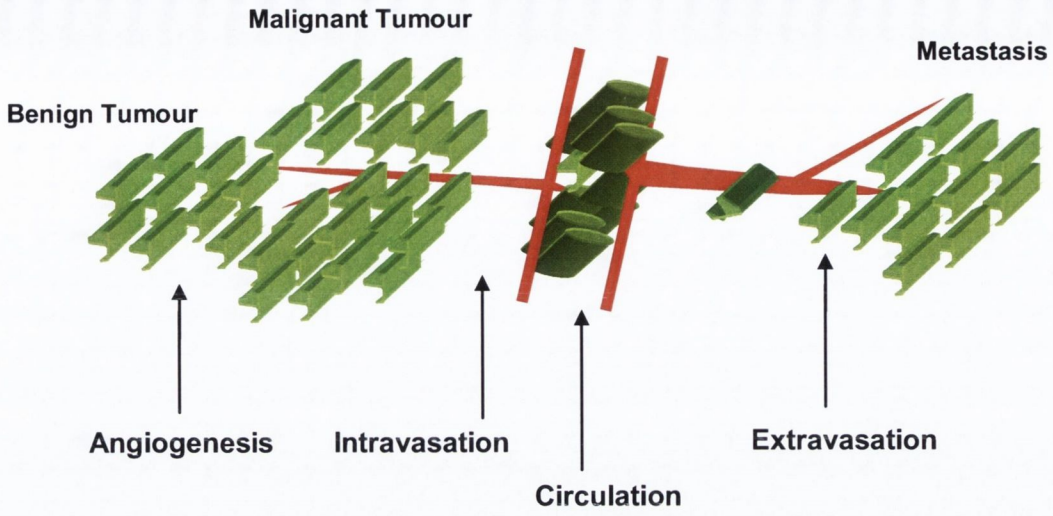


Fig. 1. Stages of cancer progression involving vascular system.

Metastasis can occur through different mechanisms such as body cavities or vascular way. The latter possibility may involve lymphatic or haematogenous spread (**Fig.1**). When in blood, cancer cells interact with various blood elements including leukocytes and platelets.

The Platelet: Overview

In 1882 Giulio Bizzozero discovered that platelets are responsible for clot formation. These anucleate blood elements, which are formed from megakaryocytes, play an important role in vascular homeostasis. Platelets are the smallest blood element averaging 2-5 μm in diameter and 0.5 μm in thickness. They circulate in blood for 7-10 days at a concentration of $1.5 \times 10^5 - 4 \times 10^5$ platelets/μl (Tocantins 1938; Michelson 2003). Old platelets are processed by phagocytosis in the spleen and by Kupffer cells in the liver. Mitochondria, endoplasmic reticulum and other organelles or structures such as glycosomes, peroxisomes, circumferential band of microtubuli and open canalicular system are present in platelet cytoplasm (Heijnen, Debili et al. 1998;

White 1999; White 2004). Platelets contain three major types of secretory organelles such as α granules, dense bodies (δ granules) and lysosomes. To support shape of platelets, actin and myofilaments form the cytoskeleton (White 1983).

The main function of platelets is **haemostasis** and **thrombosis**. They are also natural source of growth factors. Under physiological conditions, platelets are not- active (resting) and have discoid shape, and this resting state is assured by such factors as nitric oxide (NO), prostacyclin (PGI_2), and adenosine diphospatase (ADP-ase), which are released from endothelial cells and inhibit activation of platelets (Michelson 2003) in the circulating blood. After the vascular injury, the circulating platelets are activated and recruited to repair the damage. Human platelets can be mainly activated by a number of factors including thromboxane A_2 (TXA_2) (Needleman 1976), adenosine diphosphate (ADP) (Born 1962) and matrix metalloproteinase-2 (MMP-2) (Sawicki 1997). These agents induce platelet adhesion, activation and aggregation (Nurden 1975). Of note, upon activation platelets change their shape, form pseudopodia (**Fig. 2**) and release growth factors.

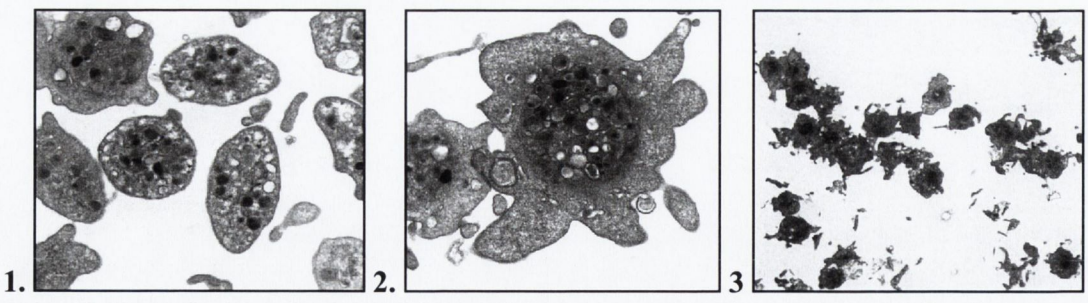


Fig. 2. TEM images of human platelets. 1. Resting platelets as shown by their discoid shape; 2. Activated platelets. Note granular centralization and formation of pseudopodia; 3. Aggregated platelets.

The outer surface of the platelet membrane is very rich in glycoproteins and is known as glycocalyx. The glycoproteins receptors are necessary to facilitate

adhesion, activation and aggregation of platelets. The glycoprotein GPIb/IX/V and GPIIb/IIIa complexes are the principal mobile receptors on platelets. The outside membrane surface in resting platelets is covered by about 25.000 GPIb/IX/V and 80.000 GPIIb/IIIa receptor copies (White 1987).

Firstly, the GPIb/IX/V complex interacts with von Willebrand factor (vWF). Following damage occurring to the blood vessel, the vWF binds to the subendothelial collagen. vWF plays a major role in blood coagulation and it is important in platelet adhesion to wound sites (Sadler 1998). The GPIb/IX/V complex includes the GPIbα subunit, which is essential for vWF biding. GPIbβ and GPIX are responsible for assembling and anchoring the complex to the platelet surface. Furthermore, on the platelet surface there are two main collagen receptors such as GPIa/IIa and GPVI. The main function of GPIa/IIa is to facilitate the bonds between platelets and collagen. GPVI acts as a signalling molecule and fully activates platelets (Clemetson 2001).

Secondly, the binding of vWF to GPIb/IX/X complex leads to the release and activation of GPIIb/IIIa (Chen and Lopez 2005; Rivera, Lozano et al. 2009). In addition, when the platelets are active they translocate P-selectin (transmembrane adhesion receptor), which is mainly stored in α-granules to the platelet membrane surface (Stenberg, McEver et al. 1985; Larsen, Celi et al. 1989). The activation of these receptors leads to change in the shape of platelets. Also upon activation, platelets release growth factors and growth regulators such as growth factors, interleukin 6 (IL-6), thrombin, fibrinogen (Amirkhosravi 1999) and angiostatin (Jurasz, Alonso-Escolano et al. 2003; Jurasz, Santos-Martinez et al. 2006).

Finally, the circulating soluble fibrinogen and activated platelets form a haemostatic plug that is reinforced by the generation of fibrin by the coagulation cascade, thus forming thrombus (Fullard 2004).

In addition to haemostasis and thrombosis, platelets play also a role in non-haemostatic processes such as innate immune response, wound repair and, as mentioned before, in carcinogenesis (Jurasz, Alonso-Escolano et al. 2004; Medina, Jurasz et al. 2006). **Figure 3** shows platelet receptors involved in the tumour cell-induced platelet aggregation process.

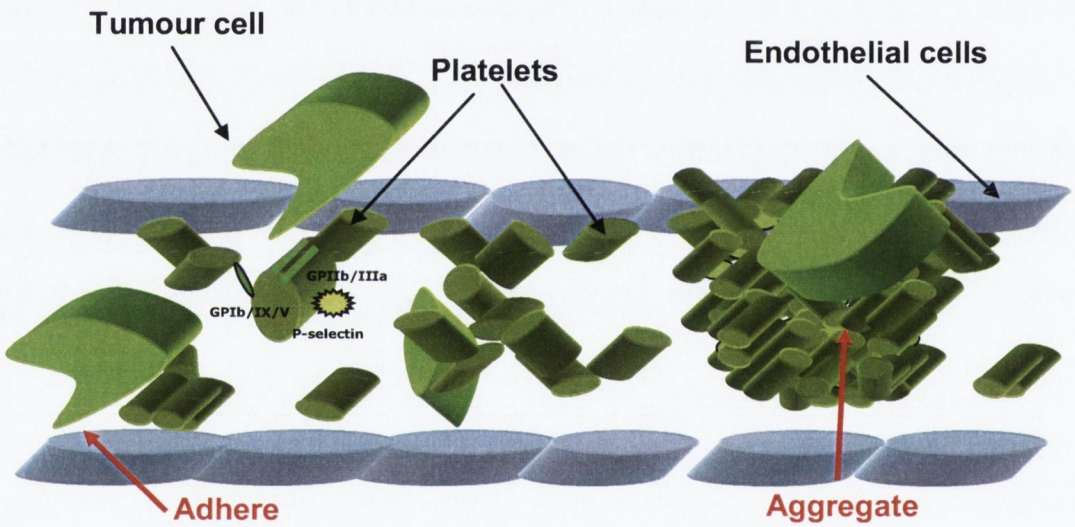


Fig. 3. Schematic representation of tumour cell-induced platelet aggregation (TCIPA) and platelet receptors involved in this process.

The Tumour Cell

Malignant neoplasm is a medical term for cancer. There are two different types of neoplasm (*Greek* for new growth) – localized and non-localized. Neoplasm that has only the characteristic of localized growth is classified as benign. Neoplasm with the characteristics of invasiveness and/or the capacity to metastasize is classified as malignant. The word "tumour", originally defined as

"a local swelling", is often used interchangeably with "cancer". The definition of cancer by the British oncologist Willis (Willis 1952):

"A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change."

How normal cells become malignant? This question has occupied scientists for decades. Although the genesis of cancer cells is very complex and multistage in nature, the major factors are likely to involve

- Genetic changes and
- Epigenetic factors

Genetic changes are a result of point mutations, gene amplification, chromosomal translocation or the action of certain viruses and chemical carcinogens. The activation of proto-oncogenes to oncogenes and the inactivation of tumour suppressor genes are the most common issues. The proto-oncogenes are genes, which normally control cell division, apoptosis and differentiation when converted to oncogenes by viral or carcinogen action. They also encode growth factor receptors and signal transduction proteins. The tumour suppressor genes (anti-oncogenes) are genes, which protect cells from cancer. If those genes are mutated, they lead to carcinogenesis, usually in combination with other genetic changes. Epigenetic factors are mechanisms outside the gene such as a cell's exposure to carcinogens or hormones, or genetic variations that modify a gene or its protein by methylation, demethylation, phosphorylation, or dephosphorylation.

According to Hanahan and Weinberg seven essential cellular changes must occur for cancer development (Hanahan and Weinberg 2000).

- self sufficiency in growth signals

- insensitivity to antigrowth signals
- evading apoptosis
- limitless replicative potential
- sustained angiogenesis
- tissue invasion and metastasis
- genomic instability

The first acquired capability of cancer, as discussed by authors is self-sufficiency in growth signals. Normal cells require mitogenic growth factors, which are necessary for cells proliferation. Tumour cells produce own growth factors, thereby reducing their dependence on stimulation from normal tissue microenvironment (Hanahan and Weinberg 2000).

The second acquired capability of cancer is insensitivity to antigrowth signals. Cell proliferation can be blocked by antigrowth factors in two different ways: by being forced into the quiescent G_0 phase or by being induced to enter into post-mitotic state and differentiation.

Another cancer feature is evading apoptosis. Apoptosis is a programmed cell death which is triggered by two different pathways: extrinsic and intrinsic. The cells undergoing apoptosis are characterized by membrane blebbing, shrinkage and condensation of chromatin. Cancer cells can avoid apoptosis by the mutation of p53 tumour suppressor gene (Sigal and Rotter 2000; Caino 2009). This kind of mutation is very common in over 50 % of tumours (Kaelin 1999).

The fourth acquired capability of cancer cells is a limitless replicative potential. Healthy cells may double up to 60 - 70 times before their death (Hanahan and Weinberg 2000). This limited number of cell proliferation is controlled by the telomeres serving as cellular "internal clock". Telomeres are sequences of DNA at the end of chromosomes, which become shortened due to each S phase

(Counter 1992). However, 85 – 90 % of cancer types have the ability to maintain their telomere length by upregulating expression of telomerase enzyme. The function of these enzyme is adding the hexanucleotide repeats onto the ends of telomeric DNA (Bryan and Cech 1999).

The fifth capability acquired by tumour cells is a sustained angiogenesis. The essential role of angiogenesis is to form vessels, which supply the oxygen and nutrients for cells in a tissue. Tumour cannot grow more than 1 - 2 milimeters in diameter without creating new blood vessels. There is a balance between promoters of angiogenesis such as VEGF and inhibitors such as angiostatin. Tumour has ability to change the balance between angiogenesis inducers and inhibitors (Hanahan and Folkman 1996). This mechanism is not completely understood.

The sixth acquired capability of cancer cells is invasion and metastasis. Human cancer cells have the ability to leave the primary tumour mass and colonize new tissue in a different part of the body. This feature of malignant neoplasm is the cause of 90 % of human cancer deaths (Sporn 1996). The expression of cellular adhesion molecules, integrins and extracellular proteases by cancer may facilitate the mechanism of metastasis (Albelda 1993; Crawford 1994 -1995; Forget 1999). The protease enzymes play role in degradation of the extracellular matrix and facilitate cancer cells to spread to surrounding areas.

The final ability of cancer cells is a genomic instability, which takes place in all above described cancer features. This ability also helps cancer cells become resistant to therapeutic agents.

Cancer and Apoptosis

Apoptosis: Overview

Apoptosis is a programmed cell death, and this process is crucial for such fundamental physiological processes as embryogenesis and homeostasis. Apoptosis requires energy and is characterized by individual cells death, cell shrinkage, condensation of chromatin, membrane blebbing, cell fragmentation, and phagocytosis of the dead cell. Apoptosis is also thought to limit the tumourigenic process (Bold, Termuhlen et al. 1997). When a mutation occurs in a proto-oncogene that converts it into an oncogene, a cell tries to repair the mutation; however, if it is not successful at repairing the damage, the cell will then undergo apoptosis. In contrast, tumour cells have the ability to evade apoptosis for instance by mutation in the p53 tumour suppressor gene, which control the apoptosis (Sigal and Rotter 2000). Moreover, anti- and pro- apoptotic member of the Bcl-2 family may be also mutated as exemplified by non-Hodgkin's lymphoma, small-cell lung cancer (Reed 1999) and gastrointestinal cancer (Adams and Cory 1998). There are two main pathways of apoptosis: the death receptor pathway and the death mitochondrial pathway. The **death receptor pathway** is mediated by members of the tumour necrosis factor receptor family (TNF). They activate pro-caspase 8 to an active form, which by cascade of many steps stimulates non-active pro-caspase 3 to caspase 3. The activation of the **mitochondrial pathway** leads to the release of p53 protein, which is responsible for activation of subpathway in the mitochondrion. This results in a release of p21 protein and **anti-apoptotic members** of the Bcl-2 protein family (BCL-X, BCL-W, MCL-1, and A1). This family comprises also agents that stimulate apoptosis known as **pro-apoptotic factors** (BAX, BAK,

BOK, BAD, BIK and BID). Both anti-apoptotic and pro-apoptotic factors are present at the surface of mitochondrial membrane and compete with each other in regulating apoptosis. Indeed, the anti-apoptotic factors inhibit release of cytochrome c while other members promote release of cytochrome c from mitochondria to cytosol. The released cytochrome c, makes a complex with protein apoptotic protease-activating factor-1 (Apaf-1). The complex activates pro-caspase 9 to an active form and finally results in activation of caspase 3 and 7. Caspase 3 and 7 act in cascade-like manner to stimulate DNA cleavage by DNAase into fragments of base pairs, which causes cell death (Chalah 2008). Mechanisms of apoptosis are shown in **Fig.4.** Given the complexity of apoptosis regulation it is not surprising, that carcinogenesis may be associated with alterations of expression of pro- or anti-apoptotic factors.

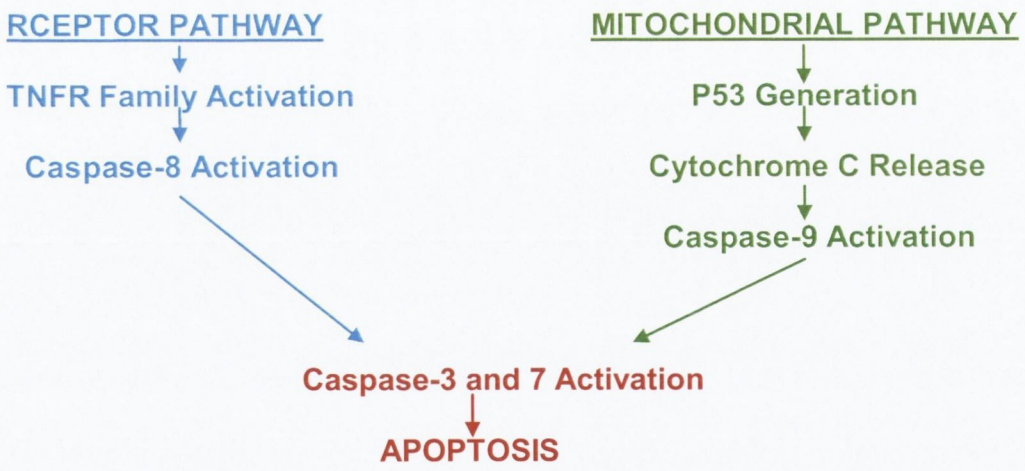


Fig. 4. Pathways of apoptosis

The cells may die not only by apoptosis but also by necrosis. Necrosis is caused by external factors, such as infection or toxins. The two mechanisms of the cells death are independent and opposite. Necrosis is characterized by large groups of cell rupture, blebbing of outer membrane and no energy is required for this process.

Cell Cycle and Cyclins

The **cell cycle** is an event which prompts the cell to duplicate into two identical daughter cells. Cell division cycle is involved in many physiological and pathological processes such as growth, repair and tumour development. There are four different phases of cell cycle G_1, S, G_2 and M (**Fig.5**).

S phase – phase of DNA synthesis

M phase – phase of mitosis, in which the cell's chromosomes and cytoplasm are divided between the two daughter cells.

G_1 phase – is the gap between mitosis and S phase, during this time the cell is preparing for DNA synthesis.

G_2 phase – is the gap between S phase and meiosis, during this time cell is preparing for the mitotic division into two identical cells.

There are two critical events during the cell cycle such as **DNA synthesis** (S phase) and **mitosis** (M phase), which entry into both of these phases is strictly regulated by **restriction points** or **checkpoints**. When DNA damage is occurred the cell cycle is stopped by the checkpoint 1 and/or 2 (Chk1, Chk2). Therefore, these checkpoints play a crucial role in genetic stability of cell. Cells may also temporarily or permanently leave the cell cycle and enter a **quiescent** or **arrested phase** known as **G_0** (Rang 2003). The duration of cell cycle phases depend on different kinds of cells. For a typical rapidly proliferating human cell with a total cycle time of 24 hours, the G_1 phase might last about 11 hours, S phase about 8 hours, G_2 about 4 hours, and M about 1 hour (Cooper 2009). The main phases of the cell cycle are shown in **Fig. 5.**

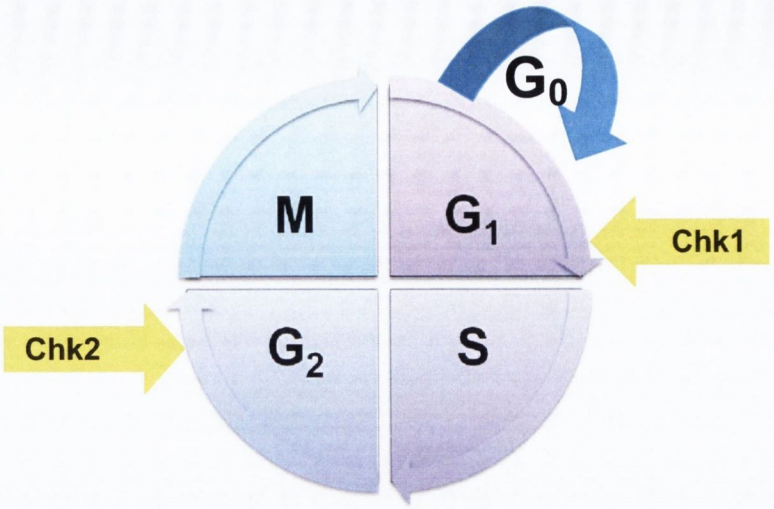


Fig. 5. Cell cycle phases.

There is a balance between positive and negative regulators of cell division.

In mammalian cells, the progression of replicating cells through the cell cycle is controlled by the sequential formation, activation, and subsequent inactivation of a series of proteins such as **cyclins** and **cyclin-dependent kinases (Cdks)** which are the **positive regulators** of cell cycle and they form together the active complex of cyclin/Cdk. This complex of proteins is degraded after they have carried out their function. The most important **cyclins**, which regulate the cell proliferation are cyclin **A**, **B**, **D** and **E**. In early stage of G_1 phase, cyclin D activates Cdk4 and/or Cdk6 and initiate phosphorylation of the retinoblastoma protein family (Rb) (Sherr and Roberts 1999). The Rb protein is binding to the E2F transcription factor, which controls the expression of the cyclins E and A gens (Lundberg and Weinberg 1998). In the late G_1 phase, Cdk2 is activated by binding to cyclin E and this leads to passage through checkpoint 1 and start DNA synthesis phase (Sherr and Roberts 2004). Then Cdk2 is binding to cyclin A forming the active complex and playing a critical role in the S period (Coverley, Pelizon et al. 2000). Complex Cdk1/cyclin A is necessary for transmitting cell to the G_2/M phase of cell cycle. Finally, Cdk1 makes the active

complex with cyclin B and completes mitosis phase (Riabowol, Draetta et al. 1989; Satyanarayana and Kaldis 2009). Both active cyclins, A and B are necessary for passing restriction point 2 (**Fig. 6**).

The **negative regulators** of the cell cycle are Rb proteins and two families of Cdks inhibitors, which are hypophosphorylated and hold the cycle in checkpoint 1. Indeed, p21WAF1, p27KIP1 and p57KIP2 belonging to the CIP family bind to cyclin/Cdk complexes. Another Ink family consist of p15, p16INK1, p18 and p19 inhibitory proteins that bind directly to Cdk4 (Draetta. 1994; Sherr 1994). The growth factors such as FGF, EGF, PDGF, VEGF and TGF-β stimulate the production of positive and negative regulators of cell cycle (Rang 2003).

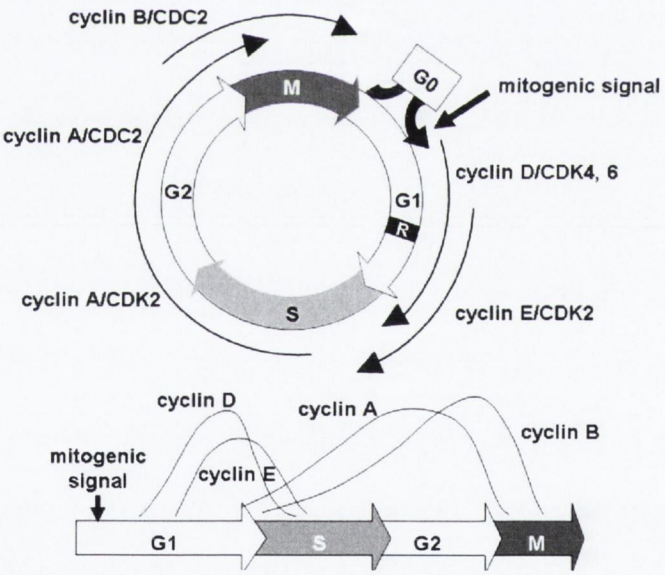


Fig. 6. Cyclin/Cdk complexes during cell cycle (Takahashi-Yanaga and Sasaguri 2008).

DNA Damage Repair Pathways

The genome of a living cell is constantly at risk of exposure to different environmental DNA damaging agents. The sources of these factors may be endogenous and exogenous, e.g. carcinogenic substances, toxins, exposure to ultraviolet (UV), ionizing radiation (IR), free radicals and chemotherapeutic agents (**Fig. 7**).

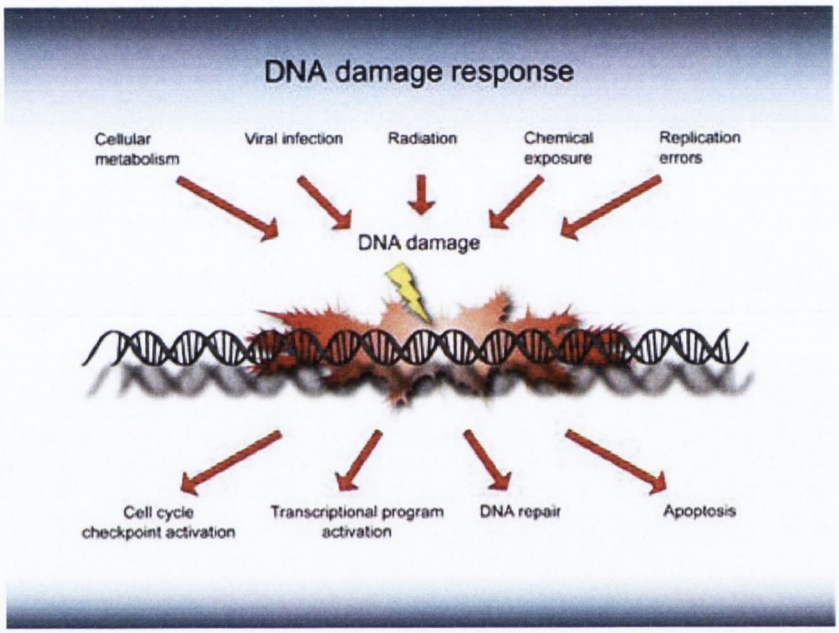


Fig. 7. DNA damage response pathways. From a Paterson Institute for Cancer Research, The University of Manchester, www.paterson.man.ac.uk/dnadamage/.

Therefore, cells evolved complex signalling networks to carefully monitor the integrity of the genome and initiate mechanisms to avoid errors such as cell cycle arrest, activation of repair pathways or apoptosis (Harper and Elledge 2007). DNA damage induces phosphorylation of H2A.X and triggers multiprotein complexes to recognize the genome defect, following activation of the transducers such as ataxia telangiectesia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR), which belong to the stress-responsive protein kinase (O'Driscoll and Jeggo 2006). After the DNA damage a series of

pathways specific signal transduction cascades are responsible for arresting the cells at the cell cycle checkpoints. Chk1 and Chk2 are checkpoint kinases downstream of ATM, ATR and DNA-PK, which play crucial role in cellular response to genotoxic stress and genome damage (Yang, Yu et al. 2003). Especially checkpoint 1 is responsible for initiating cell cycle arrest, allowing time for DNA repair and cell survival (Bartek and Lukas 2001; Zhou 2003; Ashwell and Zabludoff 2008).

There are two main pathways of DNA double strand breaks (DSBs), the **non-homologous end joining (NHEJ)** and **homologous recombination (HR)**. These two major pathways compete with each other in repairing DNA damage (Delacote, Han et al. 2002).

There are three pathways to repair single strand breaks (SSBs) such as: **base excision repair (BER)**, **nucleotide excision repair (NER)** and **mismatch repair (MMR)** (Bartek and Lukas 2001).

The **non-homologous end joining pathway** is a predominant mechanism in use for repairing DSBs during the G_0/G_1 phases. In mammalian cells NHEJ proceeds with limited end-processing by the MRN complex, which is a multisubunit nuclease composed of Mre11, Rad50 and Nbs1/Xrs2. The crucial function of the complex is contribution to DNA repair. However, recent studies have discovered role for the Mre11 complex in checkpoint signalling and DNA replication (Gottlieb and Jackson 1993; Dynan and Yoo 1998; D'Amours and Jackson 2002; Iijima 2008). Once bound to broken ends, DNA-PK is activated and phosphorylates itself and other targets including replication protein A (RPA), Werner syndrome protein (WRN), histone H2A.X and human nuclease Artemis (Burma, Chen et al. 2001; Burma and Chen 2004; Collis, DeWeese et al. 2004). One of the main proteins involved in this repair pathway is DNA-dependent

protein kinase (DNA-PK). The Ku70/80 heterodimer component of DNA-PK binds to the two DNA ends in a ring conformation. The DNA binding of Ku70/80 and aligning of the two DNA ends subsequently activates the catalytic activity of DNA-PK, which promotes the ligation of DNA ends by the XRCC4-Ligase IV complex (Ahnesorg, Smith et al. 2006; Bolderson, Richard et al. 2009). In the final step, DNA ligase IV with it is binding partner XRCC4 form a tight complex, which in the presence of XLF seals the break (Cahill 2006; Hentges, Ahnesorg et al. 2006; Shrivastav, De Haro et al. 2008).

The **homologous recombination pathway** is considered a more accurate mechanism for DSBs repair, because broken ends use homologous sequences, usually located on the sister chromatid. HR is a predominant mechanism in use for repairing DSBs during the S and G_2 phases. The proteins Rad encoded by gene Rad52 play a crucial role in this process (Johnson and Jasin 2001). This pathway initiates extensive 5' to 3' end-processing at broken ends, which is probably mediated by the Mre11/Rad50/Nbs1 complex (D'Amours and Jackson 2002). The resulting 3' single-stranded DNA (ssDNA) tails are bound by RPA, which is replaced with Rad51. Then normal base-pairing of the invading, complementary donor strands and subsequent strand are extended by DNA polymerase. The extend strand can dissociate and anneal with the processed end of the non-invading strand on the opposite side of the DSB in a process called synthesis-dependent strand annealing, or both ends may invade producing a double-Holliday junction that is resolved to yield crossover or non-crossover recombinants. Once intermediates are resolved, the remaining ssDNA gaps and nicks are repaired by DNA polymerase and DNA ligase (Bishop and Schiestl 2000; West 2003; Shrivastav, De Haro et al. 2008). BRCA1 is one of the participants during homologous recombination pathway, in which the protein

utilizes homologous intact sequence from a sister chromatid as a template. BRCA1 and BRCA2 proteins have the similar physiological function in cell. Therefore, both proteins interact with Rad51(Boulton 2006). BRCA1 colocalizes with γ-H2A.X (histone H2A.X phosphorylated on serine-139) in DNA double-strand break repair foci, indicating it may play a role in recruiting repair factors (Ye, Hu et al. 2001; Starita and Parvin 2003). The DNA double strand breaks trigger phosphorylation of H2A.X at Ser139, which can be a marker of premalignant lesions. Moreover, γ-H2A.X histon attracts the DNA repair proteins to the damaged chromatin (Jun and Anton 2008; Kinner, Wu et al. 2008; Podhorecka 2009).

The **base excision repair (BER) pathway** recognizes and removes small, non-helix base lesions, that have been damaged by oxidation, alkylation, ring saturation and ionizing radiation (Chan, Zhang et al. 2006). DNA glycosylases form AP sites (abasic site) location in DNA, that has neither purine nor pyrimidine base. These are then cleaved by an AP endonuclease. The resulting single-strand break can be repaired by either short-patch or long-patch BER (Liu, Prasad et al. 2007).

The next repair pathway of SSBs is **nucleotide excision repair** which is activated by bulky DNA lesion resulted in the damage by UV and DNA-alkylating agents (Hanawalt 2002). The short single-stranded genome disruption is removed and the single-strand gap is subsequently filled in by DNA polymerase, which uses the undamaged strand as a template. NER can be divided into two subpathways Global genomic NER and Transcription coupled NER (Cleaver 2005).

The **mismatch repair** recognizes erroneous insertion, deletion and misincorporation of bases, that can arise during replication and recombination of

DNA (Iyer, Pluciennik et al. 2005). During DNA synthesis the newly synthesised strand includes a lot of errors. Therefore, the mismatch repair machinery distinguishes the newly synthesised strand from the template and by excising the wrongly incorporated base and finally replacing it with the correct nucleotide corrects the damage (Schofield and Hsieh 2003). **Figure 8** highlights DNA repair pathways.

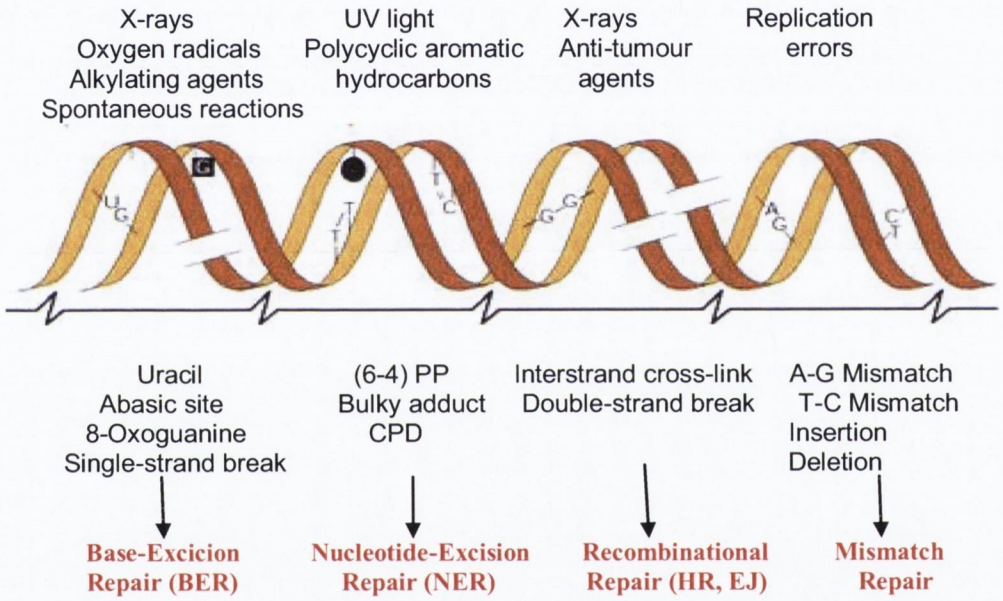


Fig. 8. DNA repair pathways (Hoeijmakers 2001).

Mitogen-activated Protein (MAP) Kinase Pathways

Mitogen-activated protein kinase (MAPK) pathways regulate a wide range of physiological processes such as differentiation, proliferation, cell survival and apoptosis. However, deregulation of MAPK activity has been implicated in several pathological situations, including inflammation, oncogenic transformation, and tumour cell invasion. There are three main pathways of MAPK: signal-regulated protein kinase (ERK 1, 2 or p42/44), c-jun N-terminal

kinases (JNK), and p38 MAPK (p38) (Pearson, Robinson et al. 2001) (**Fig. 9**). In addition, other less well-characterized MAPK pathways exist, such as the extracellular regulated kinase 5 (ERK5) pathway (Hayashi 2004). The pathways are activated by a multitude of stimuli and mediate their effects through phosphorylation. MAPK cascades are organized as modular pathways in which activation of upstream kinases by cell surface receptors leads to a sequential activation of a MAPK module (MAPKKK - MAPKK - MAPK). After MAPKs (ERK1, 2; JNK1–3 and p38 α, β, δ, γ) are activated either in the cytoplasm or in the nucleus, they bind and regulate transcription by modulating the function of a target transcription factor through serine/threonine phosphorylation (Davis 2000; Roux and Blenis 2004; Junttila, Li et al. 2008).

ERK1, 2 MAPK pathway is responsible for cell growth, cell proliferation and survival. This pathway is activated by growth factors through the g-protein Ras. Ras is a membrane-bound protein, which is activated through the exchange of bound GDP to GTP. Activated Ras recruits cytoplasmic Raf (MAPKKK) to the cell membrane for activation. There are three mammalian serine/threonine Raf kinases: A-Raf, B-Raf, and Raf-1. MEK1, 2 is activated by dual phosphorylation on two serine residues by Raf proteins. ERK1, 2 is activated by MEK1, 2, specifically by phosphorylation of a tyrosine and a threonine residue. Activated ERK1, 2 can translocate to the nucleus, where it activates several transcription factors, such as c-Fos, ATF-2, Elk-1, c-Jun, c-Myc, and Ets-1. ERK1, 2 promotes cell survival through transcriptional upregulation of anti-apoptotic Bcl-2, Bcl-xL, and Bcl-1 proteins (Bonni, Brunet et al. 1999; Ballif and Blenis 2001; Junttila, Li et al. 2008).

The **JNK (c-Jun N-terminal kinase) pathway** is activated by cellular stress and cytokines. The activation takes place through phosphorylation of a tyrosine and

a threonine residue. Physiological function of JNK pathway is to mediate apoptosis, proliferation, or survival, depending on the stimuli and cellular conditions. The pathway activates different transcription factors, such as ATF-2, Elk-1, MEF-2c, p53, and c-Myc. JNK also has other non-transcriptional substrates, for example the anti-apoptotic proteins, Bcl-2 and Bcl-xL (Davis 2000; Weston and Davis 2007; Junttila, Li et al. 2008).

The **p38 MAPK pathway** (MAPKKKs/MKK 3, 4, 6 / p38 α, β, γ, δ) is activated by the response to inflammatory cytokines and by environmental stress, such as osmotic stress, ultraviolet light, heat shock, and hypoxia. The p38 MAPK protein is represented by four isoforms: p38α, p38β, p38γ, and p38δ. Activation of all the p38 isoforms is achieved by dual phosphorylation of a threonine and a tyrosine within the threonine-glycine-tyrosine (TGY) sequence in the activation domain of the kinase (Ashwell 2006). Phosphorylated p38 proteins can activate an array of transcription factors, including ATF-2, CHOP-1, MEF-2, p53, and Elk-1. Activation of the p38 MAPK pathway is required for apoptosis leading to G_2/M cell cycle arrest. This is regulated through modulation of p53 and p73 tumour suppressor proteins (Bulavin and Fornace 2004). Conversely, p38 MAPK pathway activity has been reported to promote cancer cell growth and survival. The molecular mechanisms that determine whether p38 signalling either promotes or inhibits proliferation and survival of the cell have not been elucidated. In addition, the p38 pathway plays an essential role in regulating the expression of many inflammatory molecules, differentiation of epidermal keratinocytes, myoblasts, and immune cells (Roux and Blenis 2004; Ashwell 2006; Junttila, Li et al. 2008). **Figure 9** represents an outline of MAPK pathways.

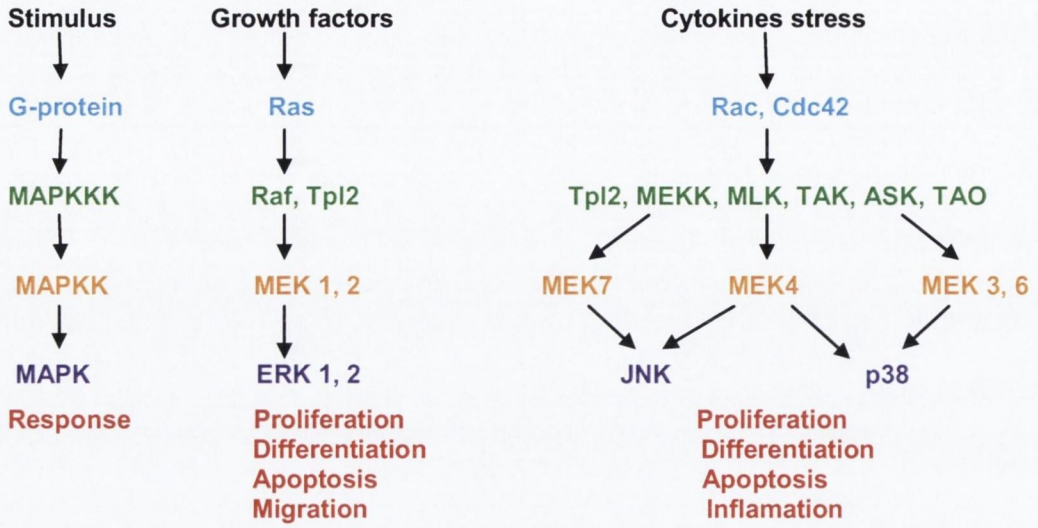


Fig. 9. Schematic outline of MAPK pathway (Dhillon, Hagan et al. 2007).

Telomere and Telomerase Activity

Telomeres are regions at the chromosomal ends of hexanucleotide sequence TTAGGG repeats, orientated towards 5'- to - 3' and a number of associated protecting and regulating proteins such as TRF1, TRF2, TIN2, POT1, TPP1 and RAP1, which collectively form a t-loop structure, which helps to maintain chromosomal integrity and stability (Griffith, Comeau et al. 1999; de Lange 2005). They play an important role in the protection and function of chromosomes. In normal somatic cells, telomeres are shortened with every cell division until the critical size is reached and cells lose their proliferative potential (Harley 1990; Harley 1997). Roughly 50 - 100 base pairs of telomeric DNA are lost with each cell cycle (Counter 1992). Maintenance of stable telomere length is associated with the activity of telomerase enzyme (**Fig. 10**). Telomerase was discovered by Carol Greider and Elizabeth Blackburn in 1985. The function of the telomerase is to add repeat sequences to the chromosomes ends while there is a loss of telomeric DNA (Morin 1989; Blasco 1997). The enzyme is ribonucleoprotein, which contains a catalytic subunit with reverse transcriptase

activity (hTERT), an RNA part that provides template for telomerase extension (hTR) and a additional telomerase associated proteins such as hTEP1, p23, Hsp90 (heat shock protein 90) and dyskerin (Blackburn 1991; Feng 1995; Nakamura, Morin et al. 1997). The human hTR is ubiquitiously expressed in all tissues, however the expression of human telomerase catalytic subunit (hTERT) is the limiting factor for telomerase activity (Keller, Brassat et al. 2009). The telomerase ribonucleic complex is associated with several other proteins. Moreover, telomerase has the ability to mediate cell survival and anti-apoptotic functions against different cytotoxic stresses (Chung 2005). Double strand breaks of DNA damage in telomeres require various DNA repair proteins and enzymes such as ataxia telangiectasia mutated (ATM), γH2A.X, 53BP1, MDC1, Ku70/80 and Mre11/Rad50/Nbs1 complex (Fagagna, Reaper et al. 2003; Zhang, Dilley et al. 2007). This response can lead to cell cycle arrest and senescence, or apoptosis (Herbig, Jobling et al. 2004; Campisi 2005). The telomerase is nearly absent in the most somatic cells. However, approximately 90% of different types of cancer cells express and upregulate this enzyme (Kim, Piatyszek et al. 1994).

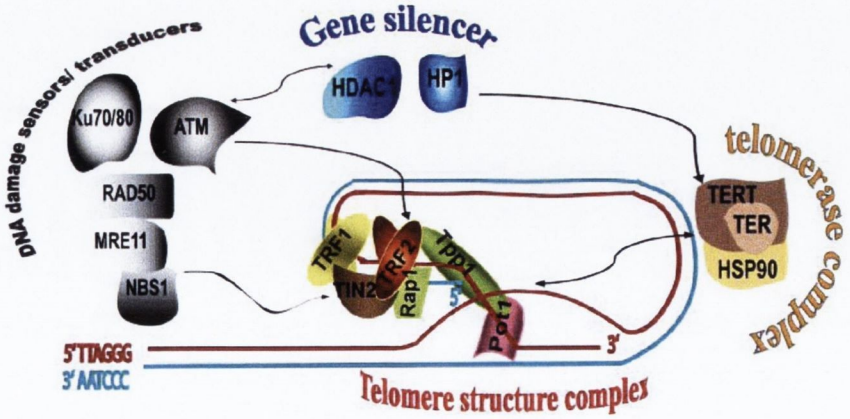


Fig. 10. Telomere and telomerase complexes (Zhang, Dilley et al. 2007).

OBJECTIVES AND AIMS

The general objective of my research was to study if platelets have the capacity to modulate cancer cell survival in response to chemotherapeutic agents.

The specific aims of this study were as follows:

1. To determine the number of live and dead human cancer cells during cancer cell damage induced by chemotherapeutic agents and the effects of platelets on these processes.
2. To investigate mechanisms involved in cancer cell-platelet interactions by studying:
    - Proteins released
    - Gene expression
    - Cell cycle and regulation of cyclins
    - DNA damage repair proteins
    - Telomerase activity

MATERIALS AND METHODS

The studies embraced by the presented thesis was approved by the Trinity College Dublin Ethics Committee.

Reagents

Prostacyclin (PGI_2), ribonuclease A (RNAse), propidium iodide, sodium azide, bovine serum albumin, Modified Eagle's Medium, Dulbecco's Modified Eagle's medium, penicillin, streptomycin, gentamicin, sodium bicarbonate, sodium pyruvate, foetal bovine serum, monoclonal anti-β-tubulin antibody produced in mouse, anti-rabbit, anti-mouse antibodies were all obtained from Sigma. Propidium iodide, annexin V APC, cyclin A, mouse IgE, cyclin B1, mouse IgG2a, cyclin D1, mouse IgG2a, cyclin E, mouse IgG1, goat anti-mouse Ig, APC conjugated were obtained from BD (Becton, Dickinson and Company). TILDA (TaqMan® Gene Expression Assays), High Capacity cDNA Reverse Transcription Kit, primers: BRC1, β-actin were obtained from Applied Biosystems. TeloTAGGG Telomerase PCR ELISA^{PLUS} kit was obtained from Roche. Millipore Immobilon Western Chemiluminescent MRP substrate was obtained from Millipore. RNA 6000 Nano Chip kit was obtained from Agilent Technologies. Phospho-BRCA1 (Ser1524) antibody, phospho-Chk1 (Ser296) antibody, phospho-histone H2A.X (Ser139), Chk1 total antibody, BRCA1 total antibody, phospho-Mre11 (Ser676) antibody, phospho-p95/Nbs1 antibody, Mre11 total antibody, p95/Nbs1 total antibody, phospho-p42/44, phospho-p38 and phospho-JNK were obtained from Cell Signalling Technology. Goat anti-rabbit HRP conjugate was obtained from BioSource.

Blood Collection, Platelet Isolation and Platelet Releasate

Blood was obtained from healthy volunteers who had not taken any drugs for 14 days prior to the study. Blood was collected in the presence of tri-sodium citrate at the final concentration of 0.315% and prostacyclin (3 μM). Washed platelet suspensions (Radomski and Moncada 1983) were isolated and resuspended (1.5×10^8 platelets per ml) in serum-free cell culture. Platelet releasate was obtained from collagen (10 μg/μl) - aggregated platelets. Platelet aggregation was measured using aggregometer (Chronolog) as the extent of light transmittance. When platelet aggregation reached maximum (90 ± 10 %) the samples were collected and releasate obtained following centrifugation of platelets at 4,500 x g for 5 minutes in the presence of prostacyclin (1 μM).

Cancer Cell Culture

Two human adenocarcinoma cell lines Caco-2 (colonic) and 59M (ovarian) were obtained from the European Collection of Cell Cultures. Cell lines were cultured as a monolayer in 75 cm^2 and 25 cm^2 culture flasks at 37 ^0C in a humidified atmosphere in the presence of 5 % CO_2.

The Caco-2 cell line was cultured in Modified Eagle's Medium (MEM) with penicillin (0.06 mg/ml), streptomycin (0.01 mg/ml), gentamicin (0.05 mg/ml), sodium bicarbonate (2.2 g/L), sodium pyruvate (0.11 g/L) and with 20 % FBS. The cell line was subcultured twice a week and supplied with fresh medium every two days.

The 59M cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with penicillin (0.06 mg/ml), streptomycin (0.01 mg/ml), gentamicin (0.05 mg/ml), sodium bicarbonate (3.7 g/L), sodium pyruvate (0.11 g/L), L-glutamine

(0.27 g/L), insulin (0.7 mg/L) and 10 % FBS. This cell line was subcultured once a week and supplied with fresh medium every two days. Cells were detached from the flask using trypsin/EDTA. All cell culture reagents were purchased from Sigma.

Cancer Chemotherapeutics

Paclitaxel supplied as 6 mg/ml, concentrate for solution for infusion (Medac UK, Actavis Ireland Limited) and 5-Fluorouracil 25 mg/ml, solution for injection (Medac UK) were obtained thanks to courtesy of the Pharmacy Department of St. James's Hospital in Dublin, Ireland.

Cancer Cell-Platelet Incubation

Platelets were added to T25-cell culture flasks containing subconfluent Caco-2 or 59M cells. Platelet-cancer cell cultures were then incubated in the presence or absence of paclitaxel (1 - 200 µg/ml) or 5-fluorouracil (0.001 - 3 mg/ml) for 1, 24 or 72 hrs. At the end of incubation conditioned media were collected and cancer cells were harvested using Trypsin/EDTA and finally washed using Binding Buffer (0.1 M Hepes, 1.4 M NaCl, 25 mM $CaCl_2$) for flow-cytometry studies.

Flow Cytometry

Apoptosis

Cancer cells apoptosis assays were performed using a Becton Dickinson flow cytometer (FACSArray, fluorescence-activated cell-sorting). Briefly, samples of incubates (20 µl) were stained with antibodies Annexin-V APC (5 µl) and

Propidium Iodide (5 µl) for 15 min in the dark at room temperature. Afterwards, samples were diluted 10 fold using Binding Buffer. Ten thousand specific events were analysed by the cytometer. The instrument was set up to measure the size (forward scatter), granularity (side scatter) and cell fluorescence. Antibody binding was measured by analyzing individual cells for fluorescence. The mean fluorescence intensity was determined after correction for cell autofluorescence. Fluorescence histograms were obtained for 10,000 individual events. Data was analyzed using Cytometer RXP software and expressed as a percentage of control fluorescence in arbitrary units.

Cell Cycle

Following incubation cells were harvested using trypsin. The cells were washed with PBS and fixed in cold 70 % ethanol (diluted in PBS) for 15 minutes at room temperature. Fixed cells were divided into the appropriate number of flow cytometry tubes, containing 4 x 10^5 (Caco-2) or 10^4 (59M) cells per test. 300 µl Propidium Iodide and 50 µl RNAse were added to the cell pellet and incubated overnight at 4 °C, protected from light. 10^4 events were analyzed by flow cytometry, using a low flow rate. The percentage of cells in the G_0/G_1, S and G_2/M cell cycle phases were quantified using ModFit LT™ software.

Cyclins

Following incubation cells were harvested using trypsin. The cells were washed twice by PBS and fixed in cold 70 % ethanol (diluted in PBS) and stored overnight at -20 °C. The fixed cells were divided into the appropriate number of flow cytometry tubes, containing 3 x 10^5 (Caco-2) and 10^4 (59M) cells per test. The cells were washed twice in PBS and 1 ml 0.25 % Triton X-100 in PBS were added to each cell pellet. The cells were mixed and incubated for 5 minutes at room temperature. Each sample was filled with staining buffer (PBS containing 1

% FCS (Foetal Calf Serum)). To the cell pellet 2.5 µl of cyclins A, B1, D1 and E antibodies (Cyclin A, mouse IgE, Cyclin B1, mouse IgG2a, Cyclin D1, mouse IgG2a, Cyclin E, mouse IgG1) were added and incubated for 60 minutes at room temperature. Following incubation, cells were washed twice in staining buffer. 100 µl of diluted in staining buffer (1 : 50) FITC-conjugated secondary antibody (Goat anti-mouse Ig, APC conjugated) was added to each cell pellet and the samples were incubated for 60 minutes at room temperature, protected from light. After incubation, cells were washed twice in staining buffer. 300 µl Propidium Iodide and 50 µl RNAse were added to the cell pellet and incubated overnight at 4 °C, protected from light. 10,000 events were analyzed by flow cytometry.

Real-time PCR

Real-time PCR (qPCR) was carried out using an Eppendorf Realplex^2 (Eppendorf) and a TaqMan Two-Step RT-PCR method. Total cellular RNA was isolated from Caco-2 and 59M cells using RiboPure kit from Ambion according to manufacturer's protocol. For reverse transcription reaction, 1 µg of total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) was used, and 10 ng of transcribed DNA was added into each qPCR reaction. As a target probe, TaqMan MGB human BRCA1 labelled with 5-carboxyfluorescein dye (Applied Biosystems) was used. As an endogenous control, TaqMan MGB eukaryotic β-actin (ACTB) ribosomal RNA probe labelled with VIC (Applied Biosystems) was used as a housekeeping gene.

TILDA (TaqMan® Gene Expression Assays)

RNA Isolation Method and Quality Control

Total cellular RNA was isolated from Caco-2 and 59M cells using miRVana kit from Ambion according to manufacturer's protocol. The quantity of RNA was measured by Nanodrop (Thermo Scientific). The quality of RNA was analyzed by Agilent 2100 Bioanalyser using the RNA 6000 LabChip® kit (**Fig. 11**).

Twelve samples were sequentially separated on a chip through a single separation channel. Each RNA chip contains an interconnected set of micro-channels that is used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. The micro-channels are filled with a sieving polymer and fluorescence dye. Agilent RNA kits are designed for use with the Agilent 2100 bioanalyzer, which electrophoretically separates the samples. The resulting data is presented as an electropherogram.

Fig. 11. A RNA Nano LabChip. From www.chem.agilent.com

TILDA

For reverse transcription reaction, 1 µg of total RNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) was used, and 100 ng of transcribed DNA (cDNA) was loaded onto the Micro Fluidic Cards.

TaqMan® Human Apoptosis Array is a micro fluidic card for quantitative gene expression analysis of targets known to have implications in programmed cell

death. This product is designed with 96 TaqMan® Gene Expression Assays loaded in singletons into the array's 384 wells and is compatible with an upgraded Applied Biosystems 7900HT Fast Real-Time PCR System. The TaqMan® Human Apoptosis Array contains assays for 93 human genes in addition to 3 endogenous controls (18S, ACTB, GAPDH). **Tables 1** and **2** show the assay map and genes symbols. The 93 genes are categorized into multiple target classes or pathways.

Target Class or Pathways

- BCL-2 Family-regulated pathway
- Death-receptor-regulated pathway
- TNF Receptor pathway
- Fas signalling pathway (CD95)
- Caspase family
- NF-κB signalling pathway
- p53 activation
- IAP family
- IAP inhibitor
- CARD family
- Kinases

Hs00236911_m1	Hs00832876_g1	Hs00180403_m1	Hs00354836_m1	Hs00892481_m1	Hs00362072_m1	Hs00169152_m1	Hs01018151_m1
Hs00209789_m1	Hs00205419_m1	Hs00376860_g1	Hs00179410_m1	Hs00215973_m1	Hs00388035_m1	Hs00223394_m1	Hs00364485_m1
Hs00169141_m1	Hs00187848_m1	Hs00154189_m1	Hs00188949_m1	Hs00261296_m1	Hs00263337_m1	Hs00154250_m1	Hs01017902_m1
Hs00368095_m1	Hs00708019_s1	Hs00248075_m1	Hs00203118_m1	Hs00219876_m1	Hs00212288_m1	Hs99999905_m1	Hs99999903_m1
Hs00236911_m1	Hs00832876_g1	Hs00180403_m1	Hs00354836_m1	Hs00892481_m1	Hs00362072_m1	Hs00169152_m1	Hs01018151_m1
Hs00209789_m1	Hs00205419_m1	Hs00376860_g1	Hs00179410_m1	Hs00215973_m1	Hs00388035_m1	Hs00223394_m1	Hs00364485_m1
Hs00169141_m1	Hs00187848_m1	Hs00154189_m1	Hs00188949_m1	Hs00261296_m1	Hs00263337_m1	Hs00154250_m1	Hs01017902_m1
Hs00368095_m1	Hs00708019_s1	Hs00248075_m1	Hs00203118_m1	Hs00219876_m1	Hs00212288_m1	Hs99999905_m1	Hs99999903_m1
Hs00236911_m1	Hs00832876_g1	Hs00180403_m1	Hs00354836_m1	Hs00892481_m1	Hs00362072_m1	Hs00169152_m1	Hs01018151_m1
Hs00209789_m1	Hs00205419_m1	Hs00376860_g1	Hs00179410_m1	Hs00215973_m1	Hs00388035_m1	Hs00223394_m1	Hs00364485_m1
Hs00169141_m1	Hs00187848_m1	Hs00154189_m1	Hs00188949_m1	Hs00261296_m1	Hs00263337_m1	Hs00154250_m1	Hs01017902_m1
Hs00368095_m1	Hs00708019_s1	Hs00248075_m1	Hs00203118_m1	Hs00219876_m1	Hs00212288_m1	Hs99999905_m1	Hs99999903_m1
Hs00236911_m1	Hs00832876_g1	Hs00180403_m1	Hs00354836_m1	Hs00892481_m1	Hs00362072_m1	Hs00169152_m1	Hs01018151_m1
Hs00209789_m1	Hs00205419_m1	Hs00376860_g1	Hs00179410_m1	Hs00215973_m1	Hs00388035_m1	Hs00223394_m1	Hs00364485_m1
Hs00169141_m1	Hs00187848_m1	Hs00154189_m1	Hs00188949_m1	Hs00261296_m1	Hs00263337_m1	Hs00154250_m1	Hs01017902_m1
Hs00368095_m1	Hs00708019_s1	Hs00248075_m1	Hs00203118_m1	Hs00219876_m1	Hs00212288_m1	Hs99999905_m1	Hs99999903_m1
1	2	3	4	5	6	7	8

Tab. 1. Assay Map. TaqMan® Gene Signature Array Configuration - Human Apoptosis Array. Applied Biosystems (www.appliedbiosystems.com)

Hs00154260_m1	Hs00395088_m1	Hs99999901_s1	Hs00242739_m1	Hs00172036_m1	Hs00765730_m1	Hs00174517_m1	Hs00182115_m1
Hs00230071_m1	Hs00373302_m1	Hs00223384_m1	Hs00261581_m1	Hs01057786_s1	Hs00370206_m1	Hs00559441_m1	Hs00985031_g1
Hs00234480_m1	Hs00193477_m1	Hs01847653_s1	Hs00153283_m1	Hs00153294_m1	Hs00174128_m1	Hs00175318_m1	Hs00269428_m1
Hs00989502_m1	Hs00968436_m1	Hs01042313_m1	Hs01572688_m1	Hs01063858_m1	Hs01036137_m1	Hs01043258_m1	Hs01076336_m1
Hs00154260_m1	Hs00395088_m1	Hs99999901_s1	Hs00242739_m1	Hs00172036_m1	Hs00765730_m1	Hs00174517_m1	Hs00182115_m1
Hs00230071_m1	Hs00373302_m1	Hs00223384_m1	Hs00261581_m1	Hs01057786_s1	Hs00370206_m1	Hs00559441_m1	Hs00985031_g1
Hs00234480_m1	Hs00193477_m1	Hs01847653_s1	Hs00153283_m1	Hs00153294_m1	Hs00174128_m1	Hs00175318_m1	Hs00269428_m1
Hs00989502_m1	Hs00968436_m1	Hs01042313_m1	Hs01572688_m1	Hs01063858_m1	Hs01036137_m1	Hs01043258_m1	Hs01076336_m1
Hs00154260_m1	Hs00395088_m1	Hs99999901_s1	Hs00242739_m1	Hs00172036_m1	Hs00765730_m1	Hs00174517_m1	Hs00182115_m1
Hs00230071_m1	Hs00373302_m1	Hs00223384_m1	Hs00261581_m1	Hs01057786_s1	Hs00370206_m1	Hs00559441_m1	Hs00985031_g1
Hs00234480_m1	Hs00193477_m1	Hs01847653_s1	Hs00153283_m1	Hs00153294_m1	Hs00174128_m1	Hs00175318_m1	Hs00269428_m1
Hs00989502_m1	Hs00968436_m1	Hs01042313_m1	Hs01572688_m1	Hs01063858_m1	Hs01036137_m1	Hs01043258_m1	Hs01076336_m1
Hs00154260_m1	Hs00395088_m1	Hs99999901_s1	Hs00242739_m1	Hs00172036_m1	Hs00765730_m1	Hs00174517_m1	Hs00182115_m1
Hs00230071_m1	Hs00373302_m1	Hs00223384_m1	Hs00261581_m1	Hs01057786_s1	Hs00370206_m1	Hs00559441_m1	Hs00985031_g1
Hs00234480_m1	Hs00193477_m1	Hs01847653_s1	Hs00153283_m1	Hs00153294_m1	Hs00174128_m1	Hs00175318_m1	Hs00269428_m1
Hs00989502_m1	Hs00968436_m1	Hs01042313_m1	Hs01572688_m1	Hs01063858_m1	Hs01036137_m1	Hs01043258_m1	Hs01076336_m1
9	10	11	12	13	14	15	16

Tab. 1. (Continued) Assay Map. TaqMan® Gene Signature Array Configuration - Human Apoptosis Array. Applied Biosystems (www.appliedbiosystems.com)

Hs00234431_m1	Hs00560402_m1	Hs00232399_m1	Hs00153550_m1	Hs00269492_m1	Hs00196075_m1	Hs00248187_m1	Hs00201637_m1
Hs00745222_s1	Hs00977611_g1	Hs00236330_m1	Hs00181225_m1	Hs00188930_m1	Hs00751844_s1	Hs00608023_m1	Hs00187845_m1
Hs00601065_g1	Hs00169407_m1	Hs00705213_s1	Hs00234356_m1	Hs00538709_m1	Hs00366272_m1	Hs00153439_m1	Hs00172768_m1
Hs00609632_m1	Hs00969291_m1	Hs01031947_m1	Hs99999086_m1	Hs00980365_g1	Hs01011159_g1	Hs00961847_m1	Hs01594281_m1
Hs00234431_m1	Hs00560402_m1	Hs00232399_m1	Hs00153550_m1	Hs00269492_m1	Hs00196075_m1	Hs00248187_m1	Hs00201637_m1
Hs00745222_s1	Hs00977611_g1	Hs00236330_m1	Hs00181225_m1	Hs00188930_m1	Hs00751844_s1	Hs00608023_m1	Hs00187845_m1
Hs00601065_g1	Hs00169407_m1	Hs00705213_s1	Hs00234356_m1	Hs00538709_m1	Hs00366272_m1	Hs00153439_m1	Hs00172768_m1
Hs00609632_m1	Hs00969291_m1	Hs01031947_m1	Hs99999086_m1	Hs00980365_g1	Hs01011159_g1	Hs00961847_m1	Hs01594281_m1
Hs00234431_m1	Hs00560402_m1	Hs00232399_m1	Hs00153550_m1	Hs00269492_m1	Hs00196075_m1	Hs00248187_m1	Hs00201637_m1
Hs00745222_s1	Hs00977611_g1	Hs00236330_m1	Hs00181225_m1	Hs00188930_m1	Hs00751844_s1	Hs00608023_m1	Hs00187845_m1
Hs00601065_g1	Hs00169407_m1	Hs00705213_s1	Hs00234356_m1	Hs00538709_m1	Hs00366272_m1	Hs00153439_m1	Hs00172768_m1
Hs00609632_m1	Hs00969291_m1	Hs01031947_m1	Hs99999086_m1	Hs00980365_g1	Hs01011159_g1	Hs00961847_m1	Hs01594281_m1
Hs00234431_m1	Hs00560402_m1	Hs00232399_m1	Hs00153550_m1	Hs00269492_m1	Hs00196075_m1	Hs00248187_m1	Hs00201637_m1
Hs00745222_s1	Hs00977611_g1	Hs00236330_m1	Hs00181225_m1	Hs00188930_m1	Hs00751844_s1	Hs00608023_m1	Hs00187845_m1
Hs00601065_g1	Hs00169407_m1	Hs00705213_s1	Hs00234356_m1	Hs00538709_m1	Hs00366272_m1	Hs00153439_m1	Hs00172768_m1
Hs00609632_m1	Hs00969291_m1	Hs01031947_m1	Hs99999086_m1	Hs00980365_g1	Hs01011159_g1	Hs00961847_m1	Hs01594281_m1
17	18	19	20	21	22	23	24

Tab. 1. (Continued) Assay Map. TaqMan® Gene Signature Array Configuration - Human Apoptosis Array. Applied Biosystems (www.appliedbiosystems.com)

BIRC2	BAK1	BCL3	CASP1	CASP2	CASP5	CASP7	CASP8
BCL2L13	TNFRSF21	HTRA2	TBK1	ESRRBL1	LRDD	CARD15	CARD9
BCL2L1	BCL2L2	BIK	BNIP3L	BOK	CASP3	CASP6	CASP10
BCL2L10	BCL2L11	BBC3	PYCARD	DIABLO	BIRC6	GAPDH	ACTB
BIRC2	BAK1	BCL3	CASP1	CASP2	CASP5	CASP7	CASP8
BCL2L13	TNFRSF21	HTRA2	TBK1	ESRRBL1	LRDD	CARD15	CARD9
BCL2L1	BCL2L2	BIK	BNIP3L	BOK	CASP3	CASP6	CASP10
BCL2L10	BCL2L11	BBC3	PYCARD	DIABLO	BIRC6	GAPDH	ACTB
BIRC2	BAK1	BCL3	CASP1	CASP2	CASP5	CASP7	CASP8
BCL2L13	TNFRSF21	HTRA2	TBK1	ESRRBL1	LRDD	CARD15	CARD9
BCL2L1	BCL2L2	BIK	BNIP3L	BOK	CASP3	CASP6	CASP10
BCL2L10	BCL2L11	BBC3	PYCARD	DIABLO	BIRC6	GAPDH	ACTB
BIRC2	BAK1	BCL3	CASP1	CASP2	CASP5	CASP7	CASP8
BCL2L13	TNFRSF21	HTRA2	TBK1	ESRRBL1	LRDD	CARD15	CARD9
BCL2L1	BCL2L2	BIK	BNIP3L	BOK	CASP3	CASP6	CASP10
BCL2L10	BCL2L11	BBC3	PYCARD	DIABLO	BIRC6	GAPDH	ACTB
1	2	3	4	5	6	7	8

Tab. 2. Gene Symbols. TaqMan® Gene Signature Array Configuration - Human Apoptosis Array. Applied Biosystems (www.appliedbiosystems.com)

CASP9	IKBKB	18S	LTB	MCL1	NFKB1	NFKB2	NFKBIB
NFKBIZ	BCL2L14	BIRC7	CARD6	BIRC8	DEDD2	APAF1	BIRC3
DAPK1	HIP1	BIRC1	NFKBIA	RELA	TNF	IKBKG	PEA15
CHUK	REL	TNFRSF1A	RIPK2	IKBKE	BCAP31	ICEBERG	TA-NFKBH
CASP9	IKBKB	18S	LTB	MCL1	NFKB1	NFKB2	NFKBIB
NFKBIZ	BCL2L14	BIRC7	CARD6	BIRC8	DEDD2	APAF1	BIRC3
DAPK1	HIP1	BIRC1	NFKBIA	RELA	TNF	IKBKG	PEA15
CHUK	REL	TNFRSF1A	RIPK2	IKBKE	BCAP31	ICEBERG	TA-NFKBH
CASP9	IKBKB	18S	LTB	MCL1	NFKB1	NFKB2	NFKBIB
NFKBIZ	BCL2L14	BIRC7	CARD6	BIRC8	DEDD2	APAF1	BIRC3
DAPK1	HIP1	BIRC1	NFKBIA	RELA	TNF	IKBKG	PEA15
CHUK	REL	TNFRSF1A	RIPK2	IKBKE	BCAP31	ICEBERG	TA-NFKBH
CASP9	IKBKB	18S	LTB	MCL1	NFKB1	NFKB2	NFKBIB
NFKBIZ	BCL2L14	BIRC7	CARD6	BIRC8	DEDD2	APAF1	BIRC3
DAPK1	HIP1	BIRC1	NFKBIA	RELA	TNF	IKBKG	PEA15
CHUK	REL	TNFRSF1A	RIPK2	IKBKE	BCAP31	ICEBERG	TA-NFKBH
9	10	11	12	13	14	15	16

Tab. 2. (Continued) Gene Symbols. TaqMan® Gene Signature Array Configuration - Human Apoptosis Array. Applied Biosystems (www.appliedbiosystems.com)

NFKBIE	PMAIP1	RELB	TNFRSF1B	TNFRSF10A	CARD4	NALP1	CASP14
BIRC4	BIRC5	FAS	FASLG	BAD	BAX	BCL2	BCL2A1
TRADD	RIPK1	HRK	TNFSF10	FADD	TNFRSF10B	CFLAR	DEDD
BID	BNIP3	CASP4	LTA	TNFRSF25	CRADD	BCL10	CASP8AP2
NFKBIE	PMAIP1	RELB	TNFRSF1B	TNFRSF10A	CARD4	NALP1	CASP14
BIRC4	BIRC5	FAS	FASLG	BAD	BAX	BCL2	BCL2A1
TRADD	RIPK1	HRK	TNFSF10	FADD	TNFRSF10B	CFLAR	DEDD
BID	BNIP3	CASP4	LTA	TNFRSF25	CRADD	BCL10	CASP8AP2
NFKBIE	PMAIP1	RELB	TNFRSF1B	TNFRSF10A	CARD4	NALP1	CASP14
BIRC4	BIRC5	FAS	FASLG	BAD	BAX	BCL2	BCL2A1
TRADD	RIPK1	HRK	TNFSF10	FADD	TNFRSF10B	CFLAR	DEDD
BID	BNIP3	CASP4	LTA	TNFRSF25	CRADD	BCL10	CASP8AP2
NFKBIE	PMAIP1	RELB	TNFRSF1B	TNFRSF10A	CARD4	NALP1	CASP14
BIRC4	BIRC5	FAS	FASLG	BAD	BAX	BCL2	BCL2A1
TRADD	RIPK1	HRK	TNFSF10	FADD	TNFRSF10B	CFLAR	DEDD
BID	BNIP3	CASP4	LTA	TNFRSF25	CRADD	BCL10	CASP8AP2
17	18	19	20	21	22	23	24

Tab. 2. (Continued) Gene Symbols. TaqMan® Gene Signature Array Configuration - Human Apoptosis Array. Applied Biosystems (www.appliedbiosystems.com)

Proteomics

The platelet-cancer cell proteome of secreted proteins (secretome) was analysed using 6-plex Tandem Mass Tag technique. Tandem Mass Tags are a unique and novel technique to label peptides for the purpose of quantitative proteomic analysis. The tags contain three different regions: a mass reporter region, a mass normaliser region and a peptide attachment (**Fig. 12**). The chemical structures of all the tags are identical but each contains heavy atoms substituted at various positions. The mass reporter and mass normaliser regions have different molecular weight in each tag. The combination of all the regions in tag have the same total molecular weights and structure (isobaric tags) (Thompson 2003; Treumann 2010). Samples that are labelled with different tandem mass tags are pooled and analysed together by LCMSMS. The relative peak intensities of the reporter ion fragments yield quantitative information (Dayon, Hainard et al. 2008).

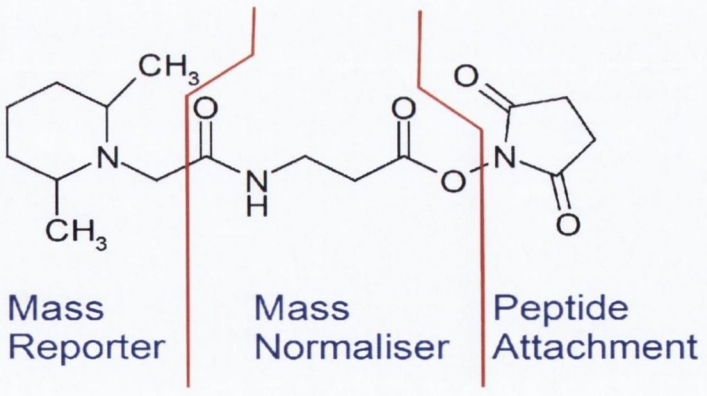


Fig. 12. Structure of the Tandem Mass Tag (Dayon, Hainard et al. 2008)

Sample

Following incubation supernatants of six co-incubates were collected and filtered by 0.45 µm Millipore filter and the Bradford assay (Protein Assay, Bio-Rad) was performed. The equivalent of 400 µg of protein was removed from each sample and protein was precipitated with 2 ml of ice cold acetone.

SDS-PAGE Separation of Proteins

Samples were redissolved with sonication in 1 X NuPage LDS sample buffer (Invitrogen), DTT was added, samples were heated for 5 min at 85 °C and separated on a 1 mm, 12 well 10%-BIS-TRIS SDS-PAGE gel (NuPage, Invitrogen). The gel was fixed in 50% MeOH, 10% CH_3COOH, H_2O (v/v/v) and stained using colloidal Coomassie Blue (EZ Blue, Sigma). Each of the six lanes was cut into 8 slices as indicated in **figure 13** and the resulting 48 samples were reduced, alkylated, *in gel* digested with trypsin and derivatized with MT reagents.

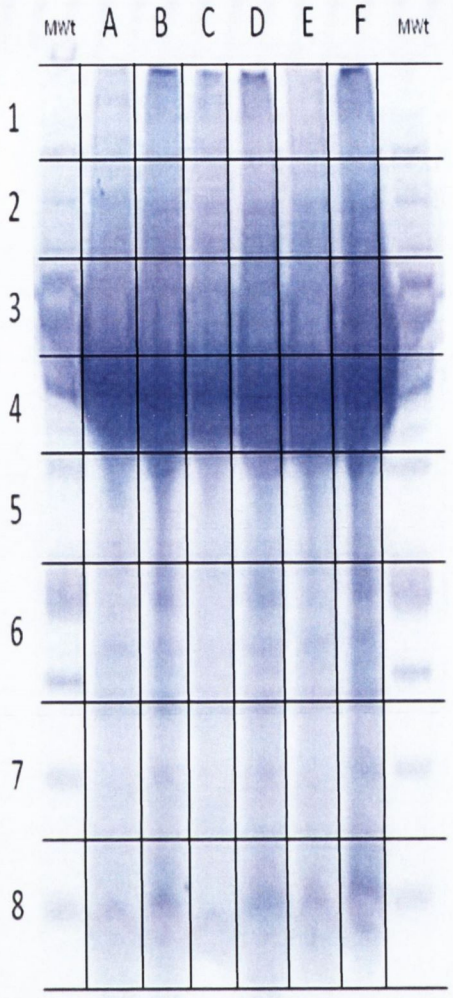


Fig. 13. SDS-PAGE separation of Caco-2 supernatants from cell culture batches treated with paclitaxel in the absence (lanes A, C and E) and in the presence (lanes B, D and F) of platelets. The gel was cut into pieces as indicated on the figure. After reduction, alkylation, *in gel* tryptic digest and labelling with TMT reagents (lane A: 126, lane B, 127, lane C, 128, lane D, 129, lane E, 130 and lane F, 131) fractions from the six different samples were combined into the pools 1 - 8 as indicated by the horizontal lines.

Reduction, Alkylation, Digestion, and Labelling with TMT of the gel slices

The 48 gel pieces were dehydrated in 100 % MeCN and rehydrated repeatedly with 50 mM TEAB (this procedure removes the Coomassie Stain that could otherwise interfere with the digest). After the last dehydration step in 100% MeCN, proteins were reduced in 10 mM DTT in 50 mM TEAB (1 h, 56 °C) and alkylated with 50 mM iodoacetamide in 50 mM TEAB (RT, dark, 45 min).

Excess reagent was removed; gel pieces were washed and dehydrated again with 70 % MeCN. Each gel piece was then digested using 8 µg of trypsin in 50 mM TEAB. After the digest, the supernatant was removed and the gel pieces were subsequently extracted with 100 µl of 50 mM TEAB (RT, 60 min) and twice with 100 % MeCN (RT, 60 min). The 48 samples were dried under vacuum until they were almost dry and subsequently 10 µl of 50 mM TEAB was added to each tube (final volume about 15 µl).

The pH in each pool was checked to make sure that it was pH 8. After performing the labelling reaction with 6-plex TMT reagents (Thermo) according to the manufacturer's instructions (lane A: 126, lane B, 127, lane C, 128, lane D, 129, lane E, 130 and lane F, 131) and quenching the reaction with 5 % hydroxylamine, samples were pooled as indicated in figure, the resulting 8 pools were dried down in a speedvac until they were almost dry and the total volume of each sample was adjusted to 30 µl using 1 % trifluoroacetic acid (TFA, Sigma).

LCMSMS analysis

LCMSMS analysis was carried out on an LTQ XL orbitrap mass spectrometer (Thermo Scientific) coupled to an Ultimate 3000 nano HPLC system. The RP-LC system consisted of a desalting column (300 µm x 5 mm, PepMap C18 3 µm, 100 Å) and an analytical column (75 µm x 250 mm, PepMap C18 3 µm, 100 Å) with split solvent delivery (split ratio 1:300). A Thermo nanospray II source was fitted with a 30 µm silica emitter tip (PicoTip, New Objective, US) and maintained at 1100V ion spray voltage. Peptide samples (5 µl) were loaded onto the trap column in 0.1 % TFA at 20 µl/min for 3 min and eluted at 300 nl/min using a gradient from 0.05 % formic acid in water (A) to 0.05 % formic acid in 80 % acetonitrile/water (v/v) (B). The gradient profile was as follows:

0 min, 4 % B; 3 min, 4 % B; 135 min, 50 % B; 136 min, 90 % B; 142 min, 90 % B; 142.1 min, 4 % B. Using Excalibur 2.0.1, intact peptides were detected between *m/z* 400 and *m/z* 2,000 in the orbitrap at a resolution of 30,000 with external calibration. Maximum ion accumulation time allowed on the LTQ orbitrap was 1s for all scan modes. Automatic gain control was used to prevent over-filling of the ion trap. Collision induced dissociation (CID) spectra of the top 3 peptide ions (rejection of singly charged precursors) were acquired normalised collision energy of 30, followed by Higher Energy C-Trap Dissociation (HCD) at a resolution of 7,500 with orbitrap acquisition. Dynamic exclusion was set with a repeat count of 2, a repeat time of 30s and an exclusion time of 3 min. The chromatography feature was enabled with a correlation area ratio of 1.0. Activation Q was set to 0.25 with 30 ms activation time.

Protein Identification and Quantitation

Using Proteome Explorer version 1.1 (Thermo), the orbitrap raw data were processed and peak lists generated from the CID spectra (for protein identification) and from the HCD spectra (for quantitation). For identification, peak lists were submitted to an in house installation of Mascot 2.2 searching the IPI HUMAN v. 3.69 database with the following parameters: MuDPIT scoring, precursor mass tolerance 10 ppm, fragment mass tolerance 0.8 Da, TMT 6-plex on peptide N-termini and Lys and carbamidomethylation on Cys as static modifications, oxidation of Met, deamidation of Asn and Gln and cyclysation of peptide N-terminal Gln to *pyro*-Glu as dynamic modifications. The false discovery rate (based on searching a decoy database) was set to 1 %.

Quantitation was carried out by ProteomeDiscoverer using 6 quantitation channels with a 20 ppm integration window around the accurate monoisotopic

masses of the 6-plex TMT reporter ions. Isotope purity correction factors for each reporter ion (rows) and the mass differences (-2,-1, 0, 1, 2) were set according to the following matrix:

0.00	0.00	92.32	7.68	0.00
0.00	0.00	93.42	6.58	0.00
0.00	0.00	94.62	5.38	0.00
0.00	1.40	94.06	4.54	0.00
0.00	0.00	96.56	3.44	0.00
0.00	1.67	94.54	3.79	0.00

Raw quantitation values were used for the subsequent statistical analysis and only unique peptides were used for the quantitation. Proteins in each channel were normalised to the protein median, resulting in the following normalisation values: (126+128+130)/(127+129+131): 0.745, 126/128: 1.005, 127/129: 0.938, 128/130: 0.646, 129/131: 0.840, 130/126: 1.43, 131/127: 1.256.

Western Blotting

Western blotting was used in order to investigate the different proteins related to DNA damage, repair and MAPK pathways. Different antibodies raised against the phosphorylated and non-phosphorylated parts of proteins BRCA1, Chk1, Mre11, p95/Nbs1, H2A.X, p38, p42/44 and JNK were used. Briefly, the samples were homogenised in homogenization buffer (Cell Extraction Buffer, BioSource) sonicated, and centrifuged at 10,000 x *g* for 20 min at 4 °C. The resultant supernatants were subjected to SDS-PAGE. Following electrophoresis, the gels were transferred to PVDF membranes (BioRad) using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system (Bio Rad). Then the blots were blocked for 1 hour in blocking buffer solution of Bovine Serum Albumin (5 % BSA) and then incubated with the different primary antibodies (2 µg/ml) overnight at 2-8 °C.

Following incubation the membranes were agitated in secondary antibody solution at room temperature for one hour. The immunoreactive bands were revealed by means of an enhanced chemiluminescence kit, (Millipore Immobilon Western Chemiluminescent MRP substrate), and the images were developed in a dark room. Densitometry was performed and analyzed using Quantity One software.

PCR-ELISA

PCR–ELISA combines PCR-amplification of labelled nucleotides and their hybridization to specific probes and hybrid capture-immunoassay in microtiter wells. This method uses the colorimetric detection (Musiani, Gallinella et al. 2007; Musiani, Venturoli et al. 2007). TeloTAGGG Telomerase PCR ELISA^{PLUS} (Roche) was used.

There are two steps of this assay (**Fig. 14**):

- **Elongation/Amplification (Telomeric Repeat Amplification Protocol, TRAP)**

P1-TS- primer was added to the biotin-labelled synthetic telomeric repeats TTAGGG of telomerase and the elongation products were amplified by PCR Eppendorf Realplex^2 (Eppendorf).

- **Detection by ELISA**

The PCR products were denatured and hybridized to a digoxigenin-(DIG)-labelled detection probes. The resulting products were immobilized via the biotin labelled primer to a streptavidin-coated microplate. These products were then detected with an antibody against digoxigenin, conjugated to horseradish

peroxidise (Anti-DIG-HRP). Finally, the probe was visualized by sensitive peroxidase substrate TMB to form a coloured reaction product.

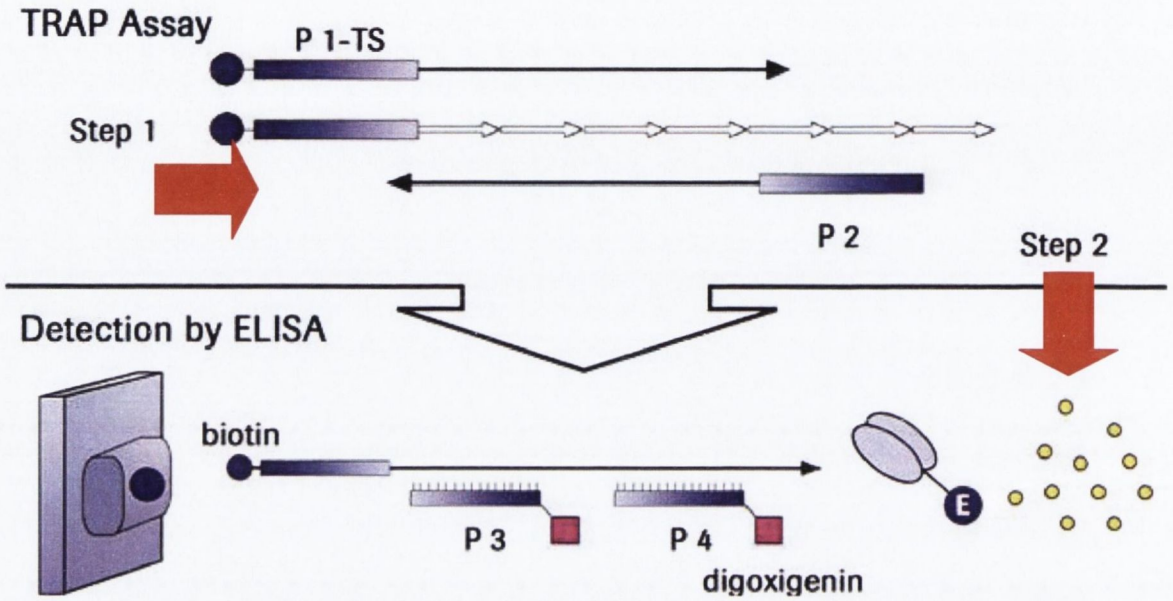


Fig. 14. Schematic outline of a PCR-ELISA assay used in this study. From www.roche-applied-science.com

Following incubation cells were harvested and 2 x 10^5 cells were used per test. The Lysis reagent of 200 µl was added to each sample and centrifuged at 16,000 x g for 20 minutes at 2 to 8 °C. Then the TRAP reaction was performed using the thermal cycler Eppendorf Realplex^2 (Eppendorf). The Denaturation reagent of 10 µl and 100 µl of Hybridization buffer T or 100 µl of Hybridization buffer IS were added to 2.5 µl of the PCR amplification product. Then 100 µl of each reaction mixture was transferred into one well of a precoated microplate module and incubated for 2 hours at 37 °C with shaking. Following shaking, the samples were removed and wells were washed three times with 250 µl of washing buffer. 100 µl of Anti-DIG-HRP solution was added to each well and incubated at 15 - 25 °C for 30 minutes while the plate was rotating. Then the samples were removed and washed five times with 250 µl of washing buffer. A solution of 100 µl 3,3',5,5'- tetramethylbenzidine was added to each well and

incubated for colour development at 15 - 25 °C for 10 - 20 minutes while the plate was rotating. Then 100 µl of Stop reagent was added to each well to stop colour development. Finally, using a microplate reader Fluostar Optima (BMG LABTECH) the absorbance of the samples was measured at 450 nm within 30 minutes after adding the Stop reagent.

Phase Contrast Microscopy

Cancer cell and cancer cell-platelet incubated were viewed using Olympus CKX41 phase-contrast microscope, equipped with an Altra 20 soft imaging system.

Statistics

The results are presented as mean ± SD calculated from n separate experiments. They were analyzed using GraphPad Prism 5 software. The results were compared using paired Student's t-test, one-way analysis of variance, repeated measures ANOVA followed by Bonferroni's or Turkey-Kramer's multiple comparison test. $P<0.05$ was considered statistically significant.

RESULTS

Effects of Platelets on Paclitaxel- Induced Apoptosis in Caco-2 and 59M Cells.

Figure 15 and **16** show flow cytometry analysis of Caco-2-platelet and 59M-platelet incubates treated with paclitaxel. The paclitaxel treatment resulted in increased number of cells undergoing apoptosis, an effect inhibited by platelets.

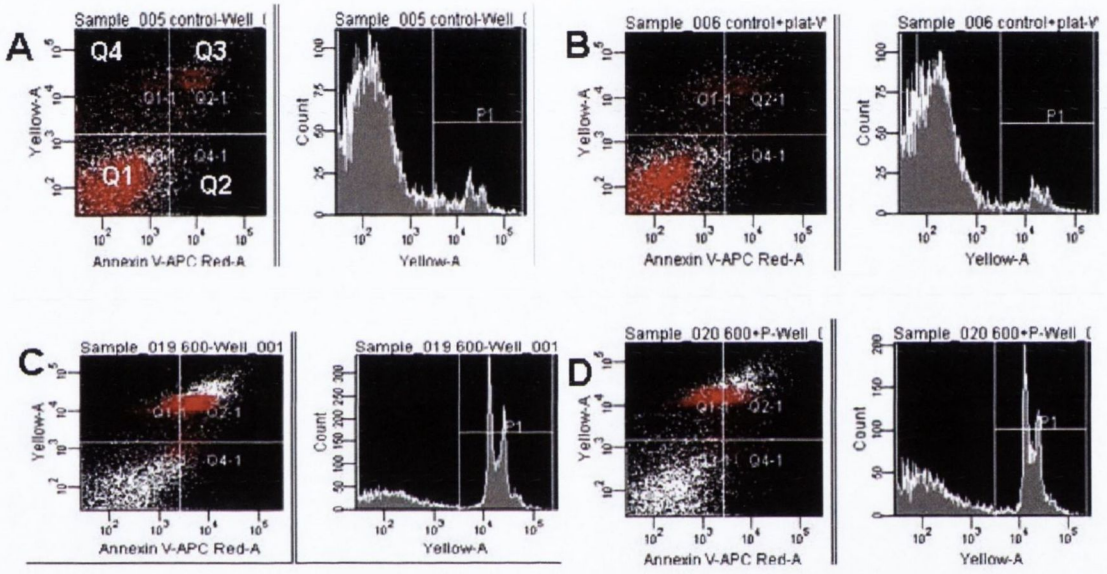


Fig. 15. Flow cytometry analysis of paclitaxel-treated Caco-2 cells for 72 hrs. Platelets protect cancer cells from paclitaxel-induced necrosis and apoptosis. Representative recordings of 5 similar experiments. A: Control incubates of Caco-2 cells in the absence of platelets. B: Incubates of Caco-2 cells with platelets. C: Incubates of Caco-2 cells in the presence of paclitaxel (200 μg/ml). D: Incubates of Caco-2 cells with platelets in the presence of paclitaxel (200 μg/ml). Q1: Live Caco-2 cells, Q2: early apoptosis, Q3: late apoptosis and Q4: necrosis.

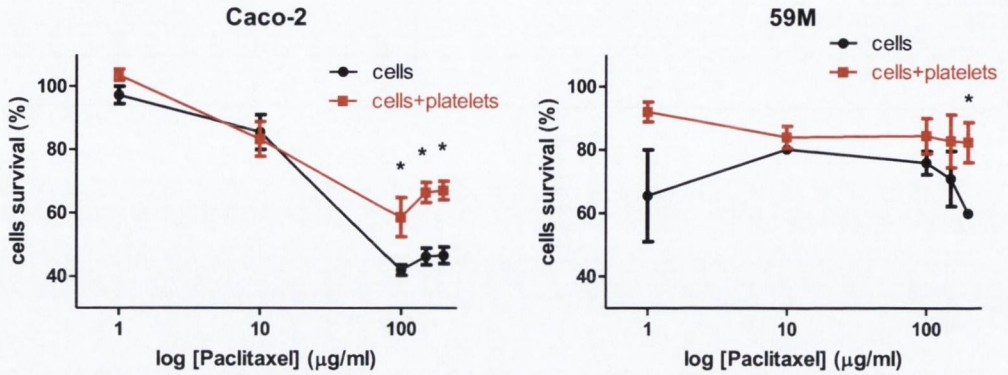


Fig. 16. Platelets protect cancer cell from paclitaxel-induced necrosis and apoptosis. Concentration-response curves showing the inhibition by platelets 1.5 x 10^8/ml of paclitaxel-induced apoptosis in 59M and Caco-2 cells. In the absence of paclitaxel the survival of 59M and Caco-2 cells was 100%. 59M cells were incubated for 24 hrs, while Caco-2 at 72 hrs. Data are mean ± SD, n=5 and n=3. *P<0.05 Caco-2 cells v/s Caco-2 + platelets and 59M cells v/s 59M + platelets.

Effects of Platelets on 5-Fluorouracil-Induced Apoptosis in Caco-2 and 59M Cells

Figure 17 and **18** show flow cytometry analysis of Caco-2-platelet and 59M – platelet incubates in the presence of 5-fluorouracil. The 5-fluorouracil treatment leads to increased number of cells undergoing apoptosis. This effect is inhibited by platelets.

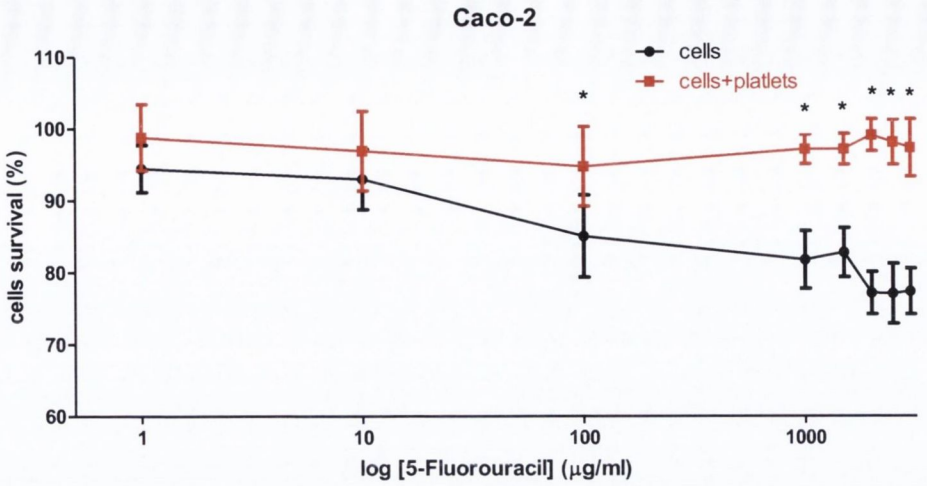


Fig. 17. Platelets protect cancer cells from 5-fluorouracil-induced necrosis and apoptosis. Concentration-response curves showing the effects of platelets 1.5 x 10^8/ml on 5-fluorouracil-induced apoptosis in Caco-2 cells. In the absence of 5-fluorouracil the survival of Caco-2 cells was 100%. Caco-2 cells were incubated for 72 hrs. Data are mean ± SD, n=4. *P<0.05 Caco-2 cells v/s Caco-2 + platelets.

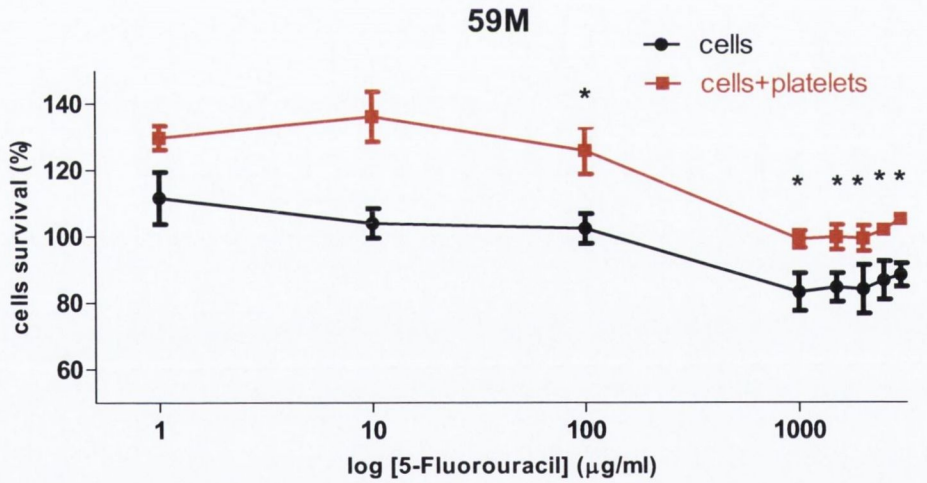


Fig. 18. Platelets protect cancer cell from 5-fluorouracil-induced necrosis and apoptosis. Concentration-response curves showing the effects of platelets 1.5 x 10^8/ml on 5-fluorouracil-induced apoptosis in 59M cells. In the absence of 5-fluorouracil the survival of 59M cells was 100%. 59M cells were incubated at 72 hrs. Data are mean ± SD, n=4. *P<0.05 59M cells v/s 59M cells + platelets.

Effects of Platelet Releasate on Paclitaxel - Induced Apoptosis in Caco-2 and 59M Cells

Figure 19 shows flow cytometry analysis of Caco-2-platelet releasate and 59M-platelet releasate incubates treated with paclitaxel. The paclitaxel treatment resulted in increased number of cells undergoing apoptosis, an effect inhibited by platelet releasate.

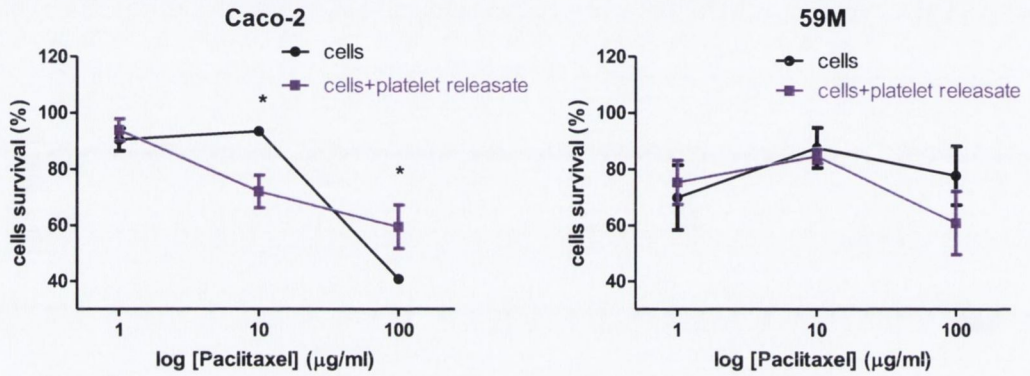


Fig. 19. Platelet releasate protect cancer cell from paclitaxel-induced necrosis and apoptosis. Concentration-response curves showing the effects of platelets 1.5 x 10^8/ml releasate on paclitaxel-induced apoptosis in 59M and Caco-2 cells. In the absence of paclitaxel the survival of 59M and Caco-2 cells was 100%. 59M cells were incubated at 24 hrs, while Caco-2 for 72 hrs. Data are mean ± SD, n=3 and n=4. *P<0.05 Caco-2 cells v/s Caco-2 + platelet releasate and 59M cells v/s 59M + platelet releasate.

Effects of Platelet Releasate on 5-Fluorouracil - Induced Apoptosis in Caco-2 and 59M Cells

Figure 20 shows flow cytometry analysis of Caco-2 - platelet releasate incubates and 59M - platelet releasate incubates in the presence of 5-fluorouracil. The 5-fluorouracil treatment leads to increased number of cells undergoing apoptosis. This effect is inhibited by platelet releasate in Caco-2 cells.

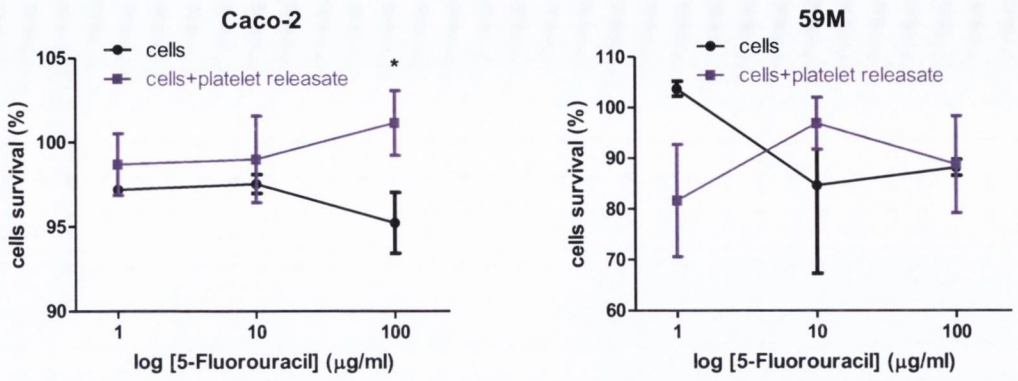
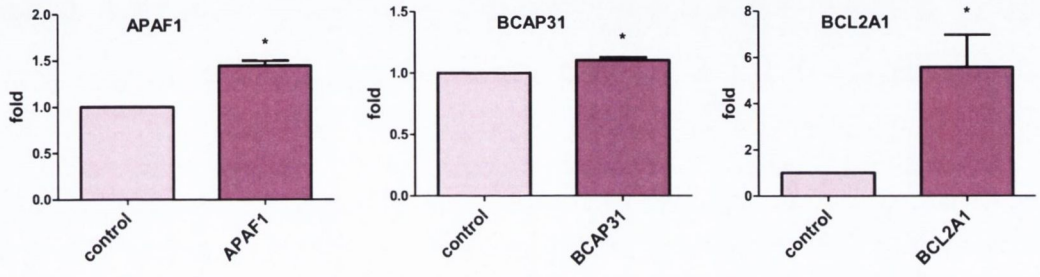


Fig. 20. Concentration-response curves showing the effects of platelets 1.5 x 10^8/ml releasate on 5-fluorouracil-induced apoptosis in Caco-2 cells and 59M. In the absence of 5-fluorouracil the survival of 59M and Caco-2 cells was 100%. Caco-2 and 59M cells were incubated at 72 hrs. Data are mean ± SD, n=3. *P<0.05 Caco-2 cells v/s Caco-2 + platelet releasate and 59M v/s 59M + platelet releasate.

Effects of Platelets on Gene Expression in 59M and Caco-2 Cells in the Presence of Paclitaxel

First, I analysed the effects of platelets on the expression of gene regulating apoptosis in control (untreated) 59M cells. **Figures 21** and **22** show that platelets resulted in upregulation of the following anti-apoptosis genes: BCL2A1, BIRC5 and the following pro-apoptotic genes: APAF1, NALP1, BCAP31, BID, CASP2, CASP8AP2, LRDD, LTB and downregulation of the following anti-apoptosis genes: CFLAR, IKBKE and pro-apoptosis genes: BCL2L11, BCL10, BNIP3L, BOK, CASP3, CRADD, PMAIP1, TNFSF10, NFKBIZ, NFKBIA, TNF1A, TA-NFKBH.



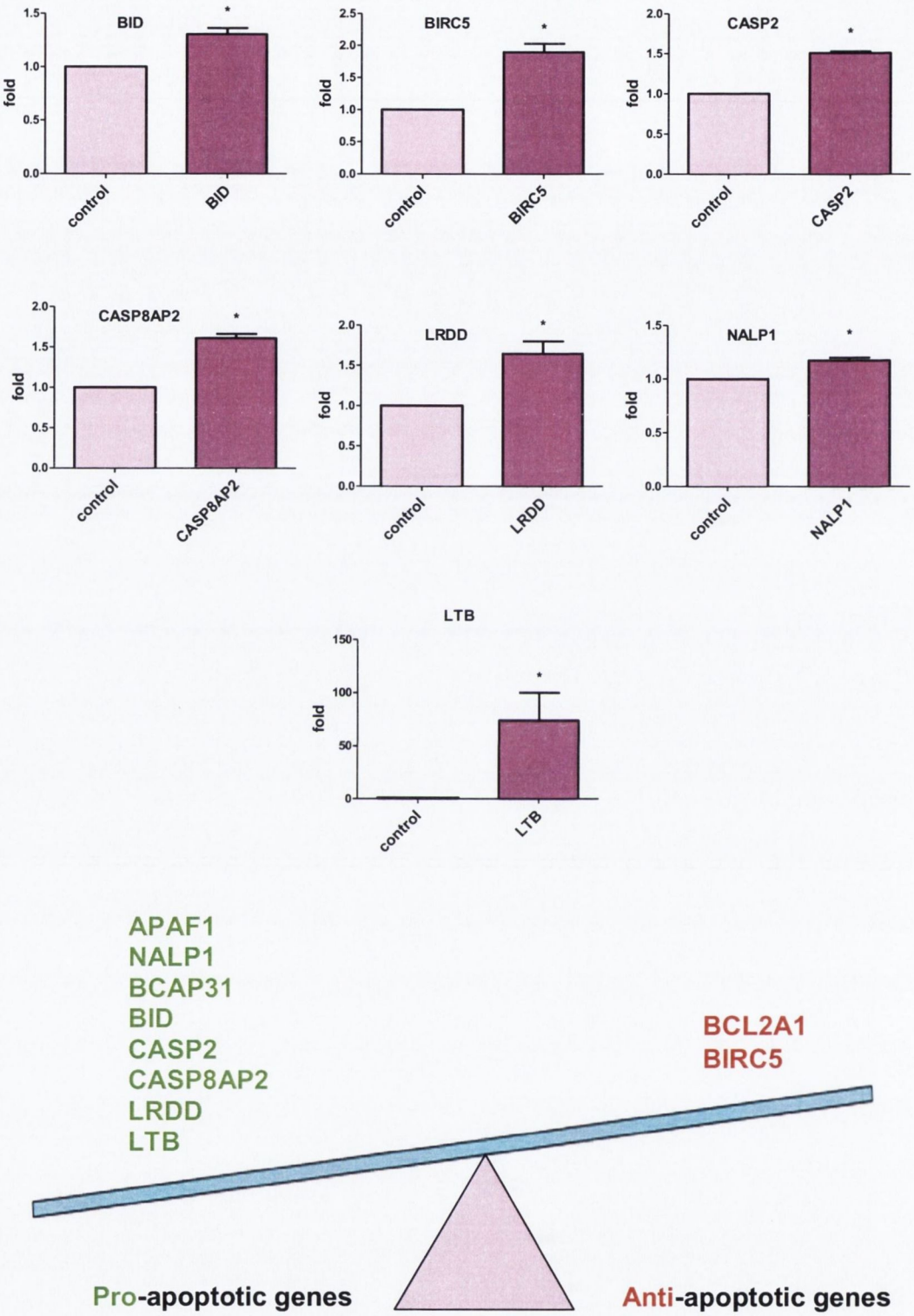
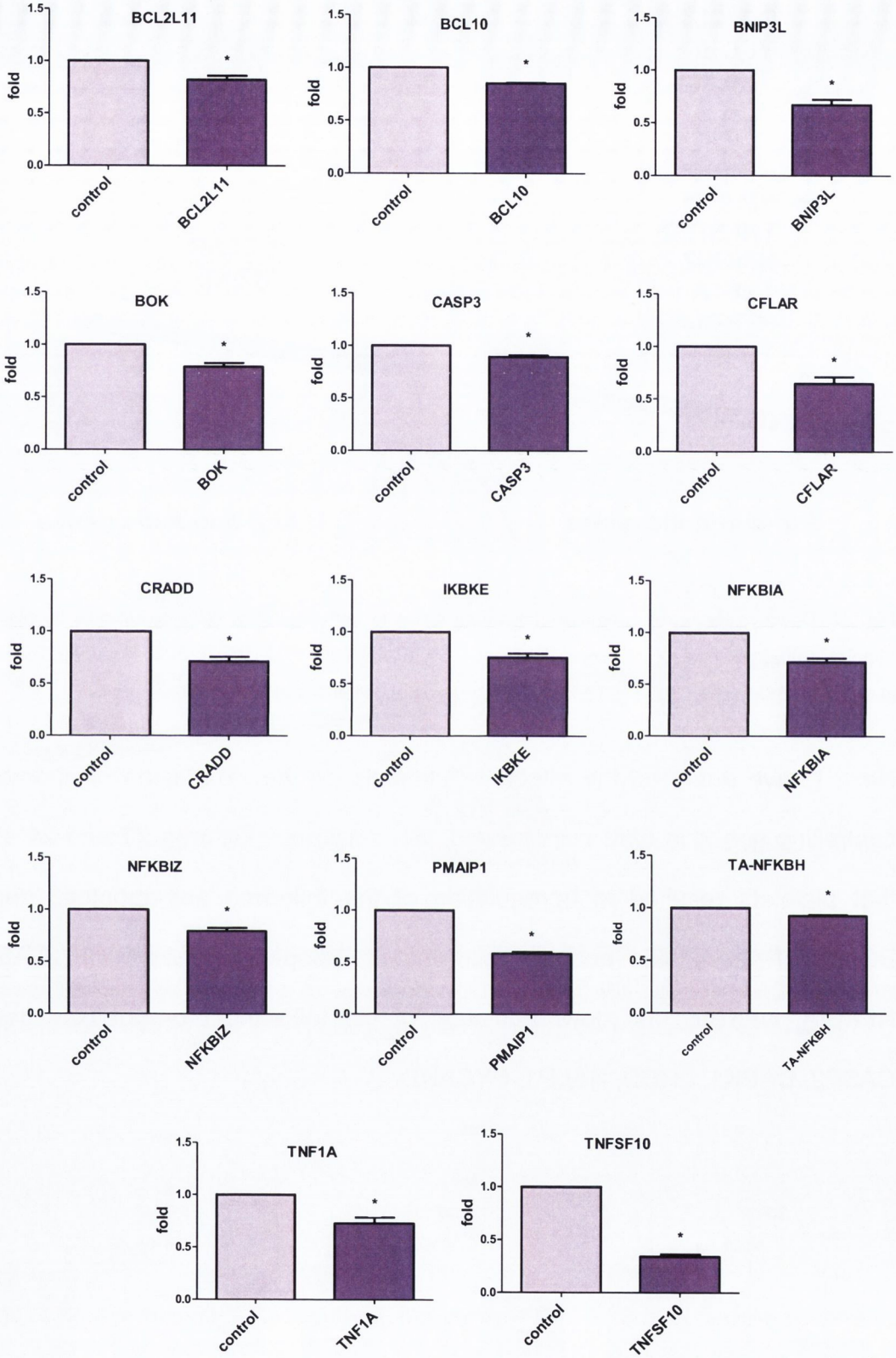


Fig. 21. Upregulation of apoptosis-controlling genes in untreated 59M cells and treated by platelets. Platelets were incubated at the concentration of 1.5 x 10^8/ml with 59M cells over 24 hrs. *P<0.05 59M + platelets v/s 59M cells, n=3. The cartoon depicts up-regulated pro- and anti-apoptosis genes.


BCL2L11
BCL10
BNIP3L
BOK
CASP3
CFLAR
CRADD
IKBKE
NFKBIA
NFKBIZ
PMAIP1
TA-NFKBH
TNF1A
TNFSF10
fold
1.5
1.0
0.5
0.0
*
control

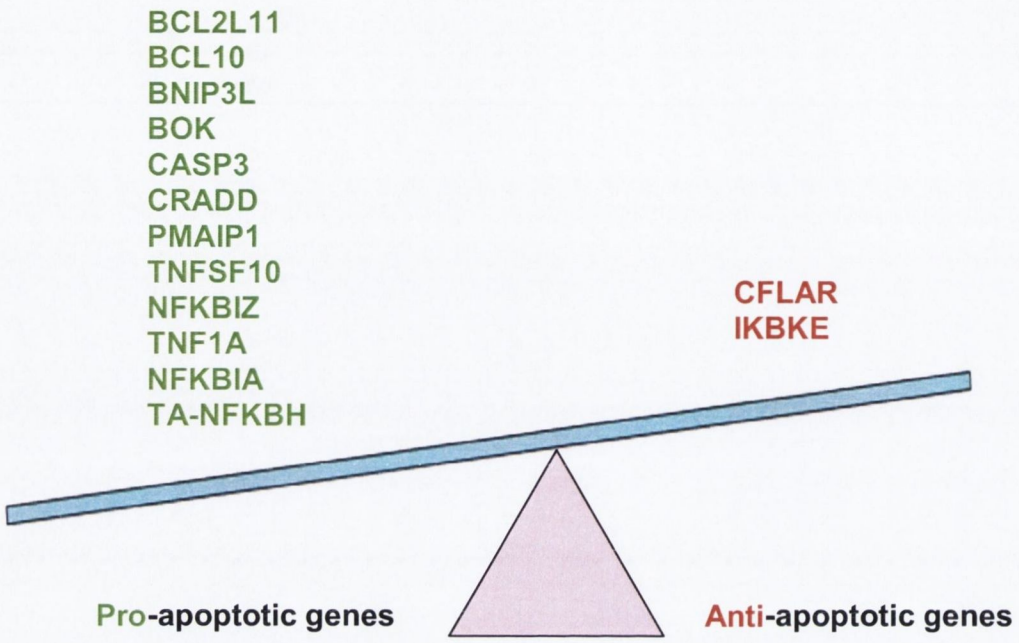
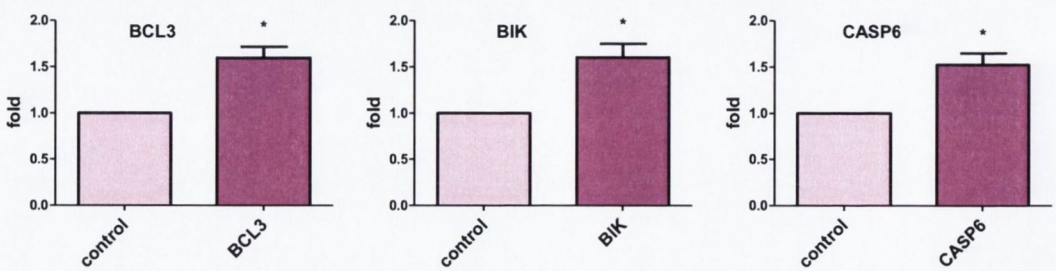


Fig. 22. Downregulation of apoptosis-controlling genes in untreated 59M cells and treated by platelets. Platelets were incubated at the concentration of 1.5 x 10^8/ml with 59M cells over 24 hrs. *P<0.05 59M + platelets v/s 59M cells, n=3. The cartoon depicts up-regulated pro- and anti-apoptosis genes

Next, I have analysed the effects of platelets on the expression of apoptosis-controlling genes in 59M cells treated with paclitaxel. **Figures 23** and **24** show that platelets resulted in upregulation of the following anti-apoptotic genes: BCL3, RIPK2, NFKB1 and the following pro-apoptotic genes: BIK, CASP6, NFKBIA, NFKBIE and downregulation of the following pro-apoptosis genes: CASP2, DAPK1, LRDD, NALP1, PYCARD.



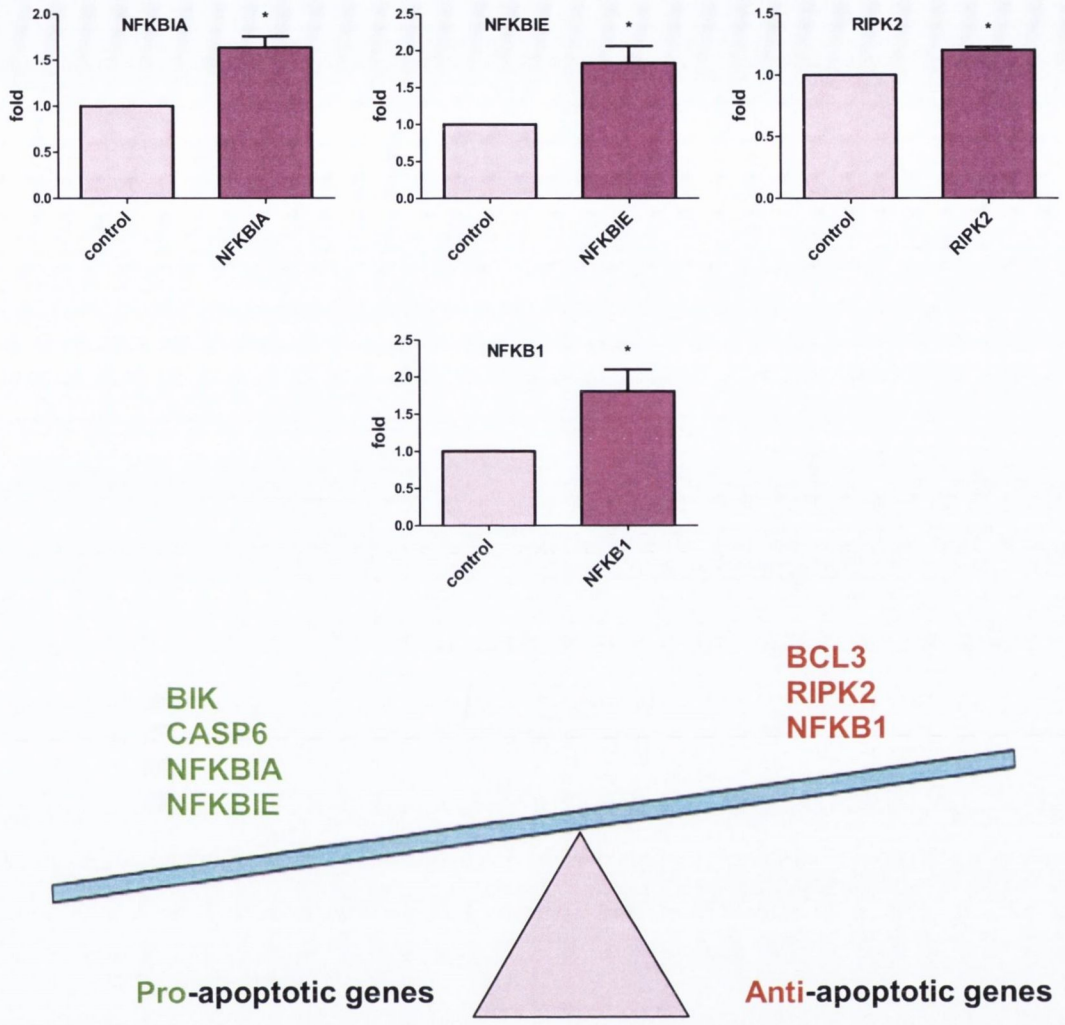
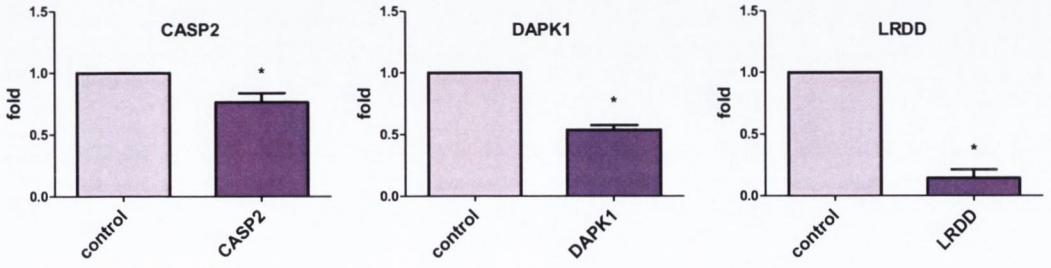


Fig. 23. Upregulation of apoptosis-controlling genes in paclitaxel (200 μg/ml) treated 59M cells by platelets. Platelets were incubated at the concentration of 1.5 x 10^8/ml with 59M cells over 24 hrs. *P<0.05 59M + platelets v/s 59M cells, n=3. The cartoon depicts up-regulated pro- and anti-apoptosis genes.



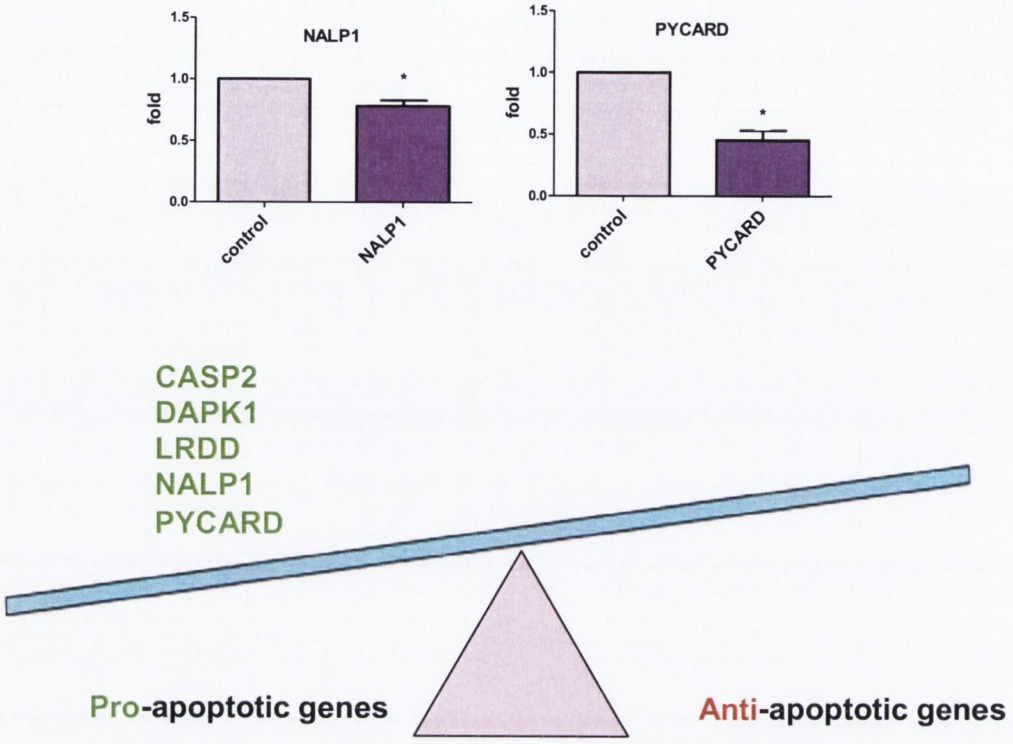
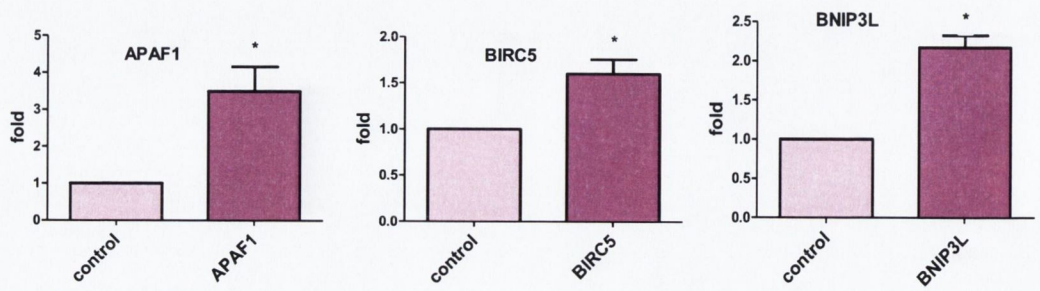


Fig. 24. Downregulation of apoptosis-controlling genes in paclitaxel-treated 59M cells by platelets. Platelets were incubated at the concentration of 1.5 x 10^8/ml with 59M cells over 24 hrs. *P<0.05 59M + platelets v/s 59M cells, n=3. The cartoon depicts up-regulated pro- and anti-apoptosis genes.

Platelets also upregulated gene expression in Caco-2 cells treated with paclitaxel. **Figures 25** shows that platelets resulted in upregulation of the following anti-apoptotic genes: IKBKG, BRIC5, NFKB2, REL and the following pro-apoptotic genes: APAF1, CASP6, BNIP3L.



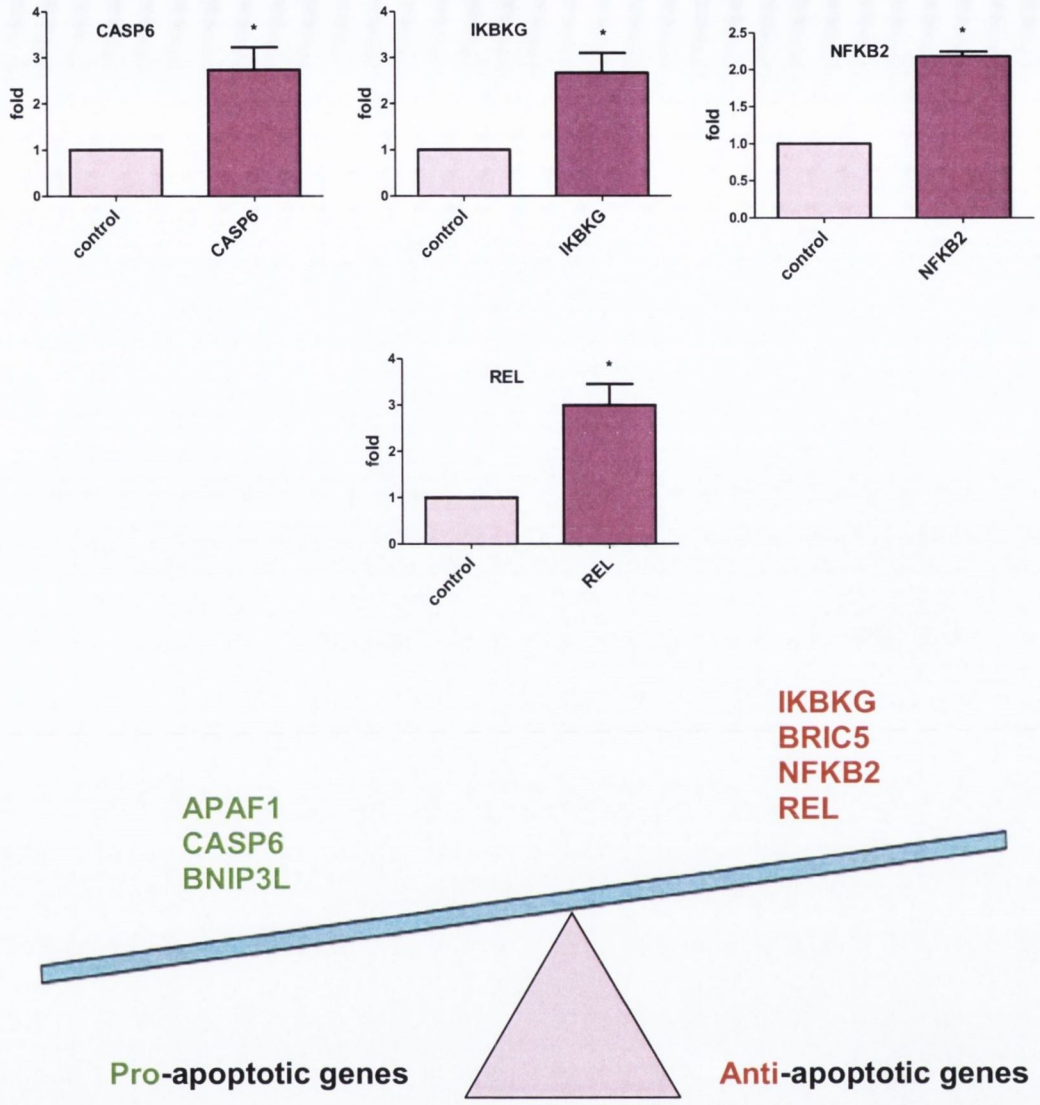


Fig. 25. Upregulation of apoptosis-controlling genes in paclitaxel (200 µg/ml) treated Caco-2 cells by platelets. Platelets were incubated at the concentration of 1.5 x 10^8/ml with Caco-2 cells over 24 hrs. *P<0.05 Caco-2 + platelets v/s Caco-2 cells, n=3. The cartoon depicts up-regulated pro- and anti-apoptosis genes.

Quality Control of RNA

Quality control is essential when quantifying gene expression experiments. The concentration and integrity of RNA samples are characterized using the Agilent 2100 bioanalyzer and RNA 6000 Nano Chip kit. **Figures 26** and **27** show the relevant quality control experiments containing reproducible data from individual

experiments. Twelve samples can be sequentially separated on a chip through a single separation channel. Data are displayed as a gel-like image as well as electropherograms.

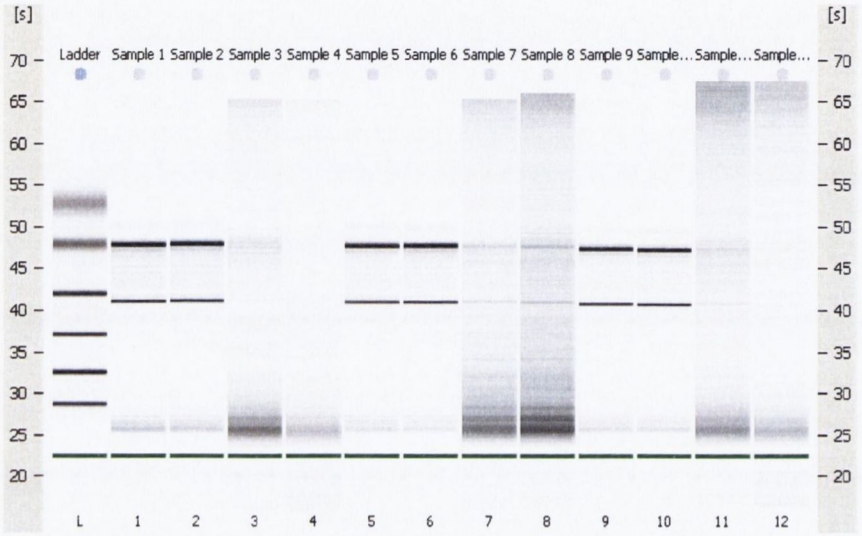


Fig. 26. The gel-like image shows the ladder and twelve samples from three repeated experiments. Samples of 59 cells after 24 hrs treatment with paclitaxel: sample 1, 5, 9 -cells; sample 2, 6, 8- cells+platelets 1.5 x 10^8/ml; sample 3, 7, 11- cells+paclitaxel 200 μg/ml; sample 4, 8, 12- cells+platelets 1.5 x 10^8/ml+ paclitaxel 200 μg/ml.

The RNA 6000 ladder is run on every chip from a specified well and is used as a reference for data analysis. The RNA 6000 ladder contains six RNA fragments ranging in size from 0.2 to 6 kb. The software automatically compares the unknown samples (1-12) to the ladder to determine the quality of the samples under standard conditions. **Figure 27** shows representative quality electropherograms of control and samples ladders of RNA 59M cells. Axis X presents migration time (s) and axis Y presents fluorescence (FU). There are two peaks in the electropherogram samples, where one of them appears shortly after 40s and another shortly before 50s. The first ribosomal RNA peak represents 18S, the second one 28S ribosomal RNA. If the 18S peak is lower

than 28S peak, the sample has the best quality. The samples with paclitaxel-treatment in 59M cells have strong effect on RNA quality, resulting in its lower quality.

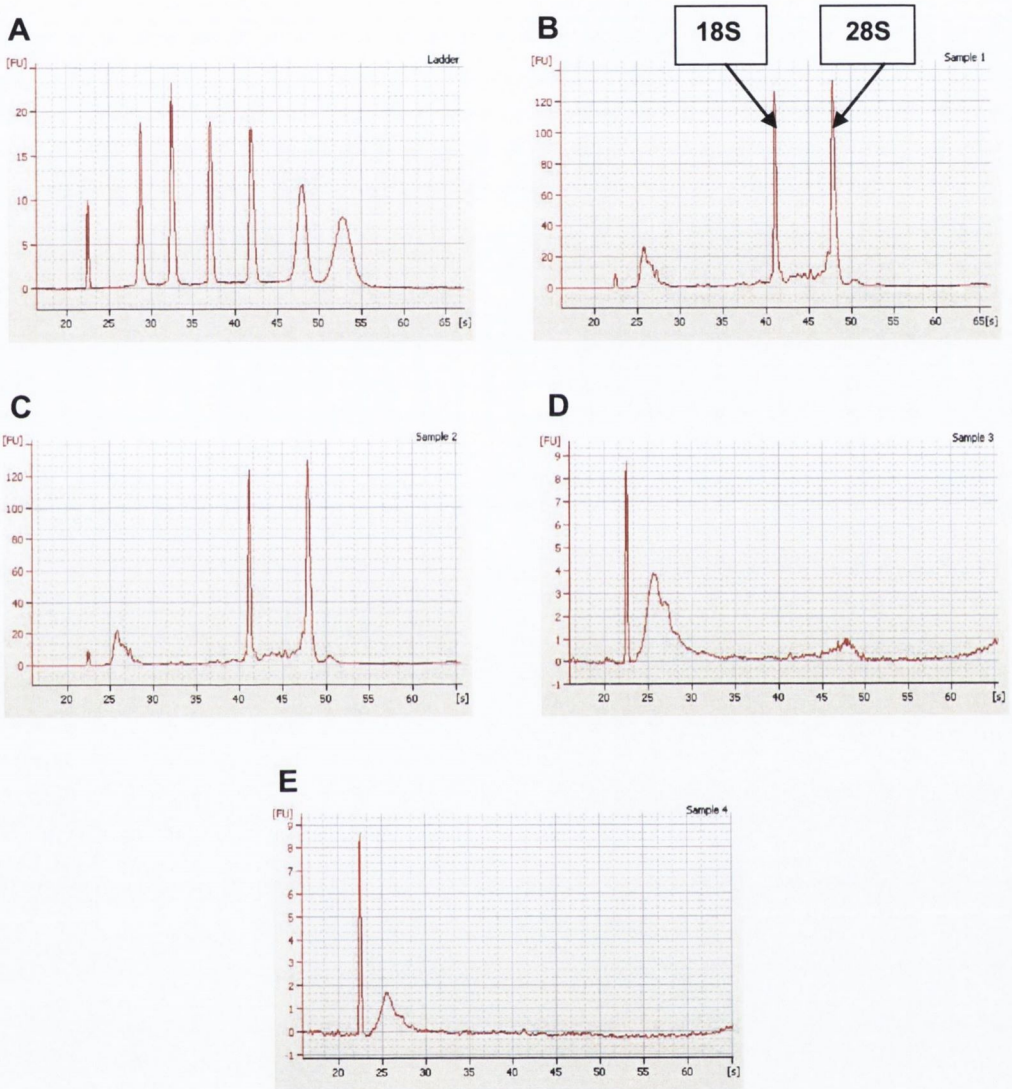


Fig. 27. Quality control of electropherograms ladders 59M cells after 24hrs treatment. A-control ladder; B-cells ladder; C-cells+platelets 1.5 x 10^8/ml ladder; D- cells+paclitaxel 200 μg/ml ladder; E-cells+platelets 1.5 x 10^8/ml + paclitaxel 200 μg/ml ladder.

Next, I have analysed the quality of Caco-2 cells RNA from three repeated experiments by using the RNA 6000 Nano Chip kit. **Figure 28** displays the data

as a gel-like image, which shows very strong effect of paclitaxel-treatment on the RNA samples.

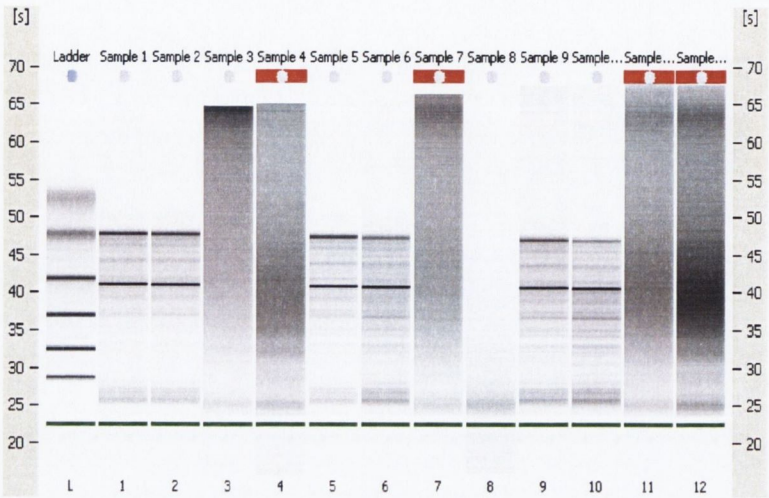
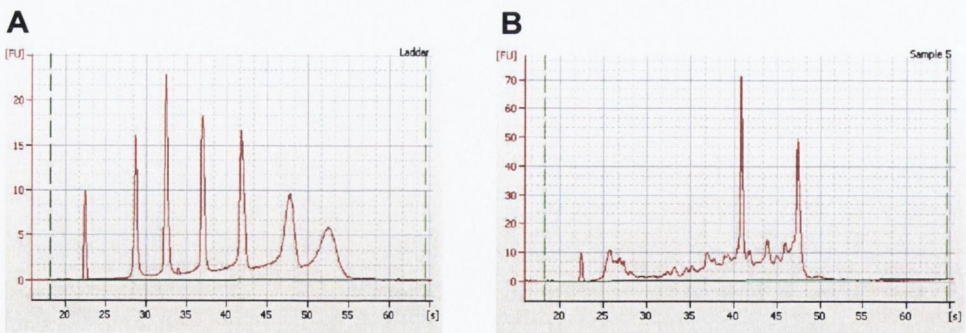


Fig. 28. The gel-like image shows the ladder and twelve samples from three repeated experiments. Caco-2 specimens after 24 hrs treatment: sample 1, 5, 9-cells; sample 2, 6, 10-cells+platelets 1.5 x 10^8/ml; sample 3, 7, 11-cells+paclitaxel 200 μg/ml; sample 4, 8, 12-cells+platelets 1.5 x 10^8/ml+ paclitaxel 200 μg/ml.

Figure 29 shows representative quality control of electropherogram ladders of RNA Caco-2 cells. The obtained data according to the ladder control indicate strong effect of paclitaxel-treatment on RNA samples, which results in lower quality of RNA.



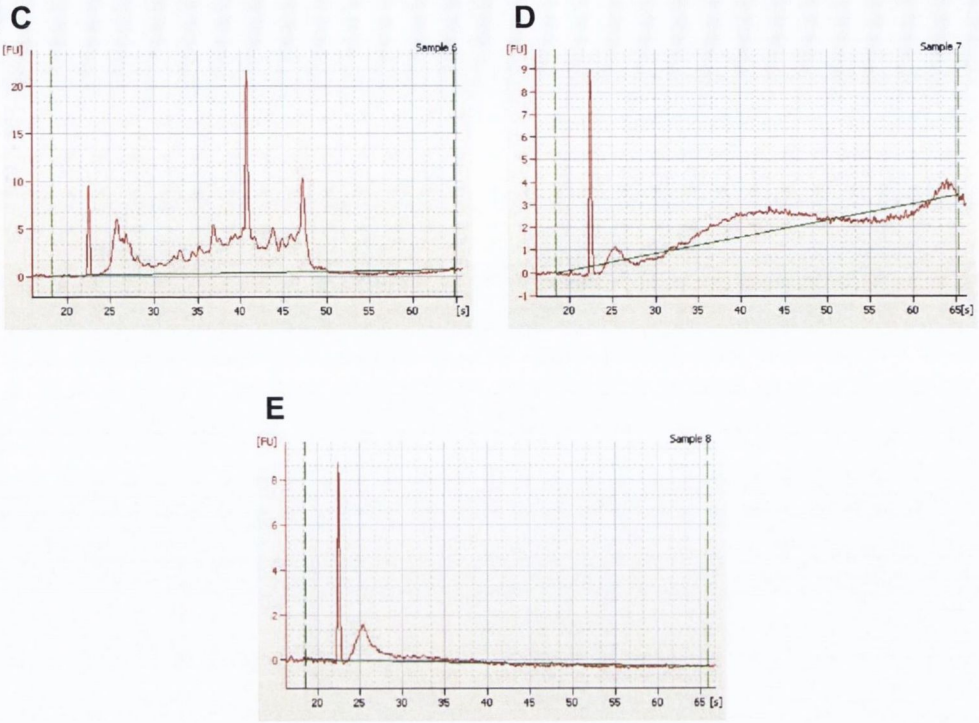


Fig. 29. Quality control of electropherogram ladders Caco-2 cells after 24hrs treatment. A- control ladder; B-cells ladder; C-cells+platelets 1.5 x 10^8/ml ladder; D-cells+paclitaxel 200 μg/ml ladder; E-cells+platelets 1.5 x 10^8/ml+ paclitaxel 200 μg/ml ladder.

TILDA experiments were performed in collaboration with ***Dr. Lorraine O'Driscoll****, School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Ireland.*

Effects of Platelets on BRCA1 Gene Expression in 59M Cells in the Presence of Platelets

I analysed the effects of platelets on the expression of BRCA1 gene, which plays essential role in repair of damaged DNA. **Figures 30** and **31** show that platelets resulted in upregulation of the BRCA1 gene in untreated and treated with paclitaxel (PTX) 59M cells.

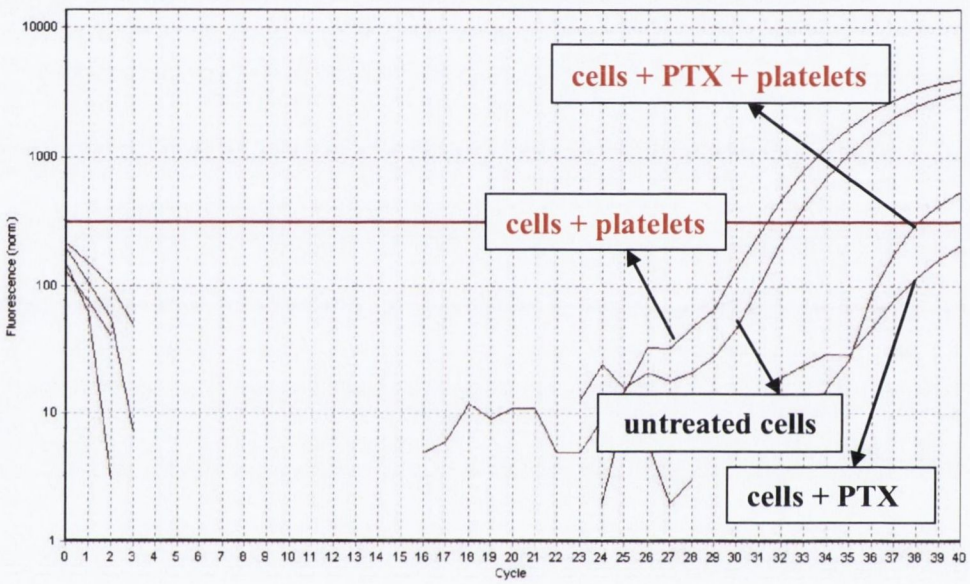


Fig. 30. qPCR analysis of untreated and paclitaxel-treated 59M cells for 24 hrs in the presence or absence of platelets 1.5 x 10^8/ml. Platelets upregulate expression of BRCA1 gene. Representative recordings of three similar experiments.

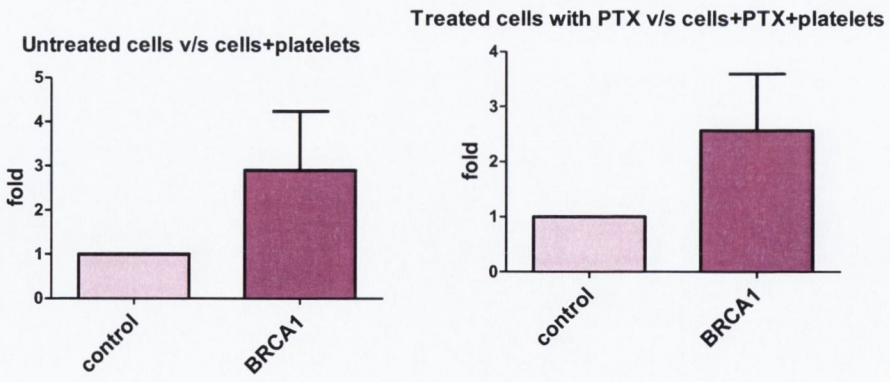


Fig. 31. Upregulation of BRCA1 gene in treated and untreated by paclitaxel (200 µg/ml) 59M cells in the presence or absence of platelets. Platelets were incubated at the concentration of 1.5 x 10^8/ml with 59M cells over 24 hrs.

Proteomics of Secretome Released During Interactions of Paclitaxel - treated Caco-2 Cells with Platelets

Table 3 shows secreted proteins during platelet - Caco-2 cells interactions in the presence of paclitaxel 200 µg/ml following 24 hrs of incubation.

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 *	trypsin inhibitor
alpha-2-HS-glycoprotein *	alpha-2-HS-glycoprotein	promotes endocytosis
haptoglobin	haptoglobin *	making the hemoglobin accessible to degradative enzymes
cutA divalent cation tolerance homolog (E. coli) *	cutA divalent cation tolerance homolog (E. coli)	forms part of a complex of membrane proteins attached to acetylcholinesterase (AChE)
thrombospondin 1	thrombospondin 1 *	adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions
ribosomal protein L23 pseudogene 8 *	ribosomal protein L23 pseudogene 8	Nucleotide binding, structural constituent of ribosome
fibrinogen alpha chain	fibrinogen alpha chain *	yielding monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 *	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	induces extensive neuronal differentiation in retinoblastoma cells and potent inhibitor of angiogenesis
Transferrin	Transferrin *	accepts electrons from ETF and reduces ubiquinone
cystatin C *	cystatin C	an inhibitor of cysteine proteinases
platelet factor 4	platelet factor 4 *	neutralizes the anticoagulant effect of heparin, chemotactic for neutrophils and monocytes, inhibits endothelial cell proliferation
glycine cleavage system protein H (aminomethyl carrier) *	glycine cleavage system protein H (aminomethyl carrier)	glycine cleavage system catalyzes the degradation of glycine
pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) *	stimulates DNA synthesis, mitosis, glycolysis, intracellular cAMP accumulation, prostaglandin E2 secretion, and synthesis of hyaluronic acid and sulfated glycosaminoglycan
peroxiredoxin 4 *	peroxiredoxin 4	involved in redox regulation of the cell, regulates the activation of NF-kappa-B

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
apolipoprotein B (including Ag(x) antigen)	apolipoprotein B (including Ag(x) antigen) *	recognition signal for the cellular binding and internalization of LDL particles by the apoB/E receptor
group-specific component (vitamin D binding protein) *	group-specific component (vitamin D binding protein)	carries the vitamin D sterols and prevents polymerization of actin by binding its monomers
apolipoprotein A-I	apolipoprotein A-I *	participates in the reverse transport of cholesterol
glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2) *	glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	plays a key role in amino acid metabolism, important for metabolite exchange between mitochondria and cytosol, facilitates cellular uptake of long-chain free fatty acids
serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 *	convert angiotensin-1 to the active angiotensin-2
lysozyme (renal amyloidosis) *	lysozyme (renal amyloidosis)	bacteriolytic function
CD9 molecule	CD9 molecule *	involved in cell adhesion, cell motility and tumour metastasis
serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7 *	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	thyroid hormone transport protein

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
complement component 3	complement component 3 *	endopeptidase inhibitor activity, receptor binding
transmembrane protein 109 *	transmembrane protein 109	the function remains unknown
integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) *	integrin alpha-V/beta-3 is a receptor for cytotactin, fibronectin, laminin, matrix metalloproteinase-2, osteopontin, osteomodulin, prothrombin, thrombospondin, vitronectin and von Willebrand factor. Integrin alpha-IIb/beta-3 is a receptor for fibronectin, fibrinogen, plasminogen, prothrombin, thrombospondin and vitronectin
proteasome (prosome, macropain) subunit, beta type, 6 *	proteasome (prosome, macropain) subunit, beta type, 6	ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH
alpha-2-macroglobulin	alpha-2-macroglobulin *	able to inhibit all four classes of proteinases by a unique 'trapping' mechanism
cadherin 17, LI cadherin (liver-intestine) *	cadherin 17, LI cadherin (liver-intestine)	calcium dependent cell adhesion protein
prolyl 4-hydroxylase, beta polypeptide	prolyl 4-hydroxylase, beta polypeptide *	breakage and rearrangement of disulfide bonds

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
inter-alpha (globulin) inhibitor H3 *	inter-alpha (globulin) inhibitor H3	a carrier of hyaluronan in serum or binding protein between hyaluronan and other matrix protein
multimerin 1	multimerin 1 *	the storage and stabilization of factor V in platelets, ligand for integrin alpha-IIb/beta-3 and integrin alpha-V/beta-3 on activated platelets
inter-alpha (globulin) inhibitor H2 *	inter-alpha (globulin) inhibitor H2	regulate the localization, synthesis and degradation of hyaluronan which are essential to cells undergoing biological processes
beta-2-microglobulin	beta-2-microglobulin *	involved in the presentation of peptide antigens to the immune system
insulin-like growth factor 2 receptor *	insulin-like growth factor 2 receptor	transport of phosphorylated lysosomal enzymes from the Golgi complex and the cell surface to lysosomes
latent transforming growth factor beta binding protein 1	latent transforming growth factor beta binding protein 1 *	controlling and directing the activity of TGFB1
serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10 *	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10	serine-type endopeptidase inhibitor activity
von Willebrand factor	von Willebrand factor *	maintenance hemostasis, adhesion of platelets to the vascular injury

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
lumican *	lumican	collagen binding, extracellular matrix structural constituent
chemokine (C-C motif) ligand 5	chemokine (C-C motif) ligand 5 *	chemoattractant for blood monocytes, memory T-helper cells and eosinophils
gelsolin (amyloidosis, Finnish type) *	gelsolin (amyloidosis, Finnish type)	calcium-regulated, actin-modulating protein
protein kinase C substrate 80K-H	protein kinase C substrate 80K-H *	regulatory subunit of glucosidase II
peroxiredoxin 3 *	peroxiredoxin 3	involved in redox regulation of the cell. Protects radical-sensitive enzymes from oxidative damage by a radical-generating system. Acts synergistically with MAP3K13 to regulate the activation of NF-kappa-B
hemopexin	hemopexin *	binds heme and transports it to the liver for breakdown and iron recovery
peroxiredoxin 5 *	peroxiredoxin 5	reduces hydrogen peroxide and alkyl hydroperoxides
apolipoprotein A-II	apolipoprotein A-II *	stabilize HDL (high density lipoprotein) structure by its association with lipids, and affect the HDL metabolism
alpha-fetoprotein *	alpha-fetoprotein	binds copper, nickel, and fatty acids as well as, and bilirubin

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
glycoprotein V (platelet)	glycoprotein V (platelet) *	GPIb-V-IX complex functions as the vWF receptor and mediates vWF-dependent platelet adhesion to blood vessels
fibronectin 1 *	fibronectin 1	involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape
glycoprotein Ib (platelet), beta polypeptide	glycoprotein Ib (platelet), beta polypeptide *	a surface membrane protein of platelets, participates in the formation of platelet plugs by binding to von Willebrand factor
prosaposin *	prosaposin	lysosomal degradation of sphingolipids
peptidylprolyl isomerase F	peptidylprolyl isomerase F *	catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides
complement C4-A Precursor (Acidic complement C4)(C3 and PZP-like alpha-2-macroglobulin domain-containing protein 2) *	complement C4-A Precursor (Acidic complement C4)(C3 and PZP-like alpha-2-macroglobulin domain-containing protein 2)	endopeptidasese inhibitor activity
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) *	caspase inhibitor activity
triggering receptor expressed on myeloid cells-like 1	triggering receptor expressed on myeloid cells-like 1 *	cell surface receptor

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
afamin	afamin *	transport
protein disulfide isomerase family A, member 6	protein disulfide isomerase family A, member 6 *	catalyzes the rearrangement of -S-S- bonds in proteins
glucosidase, alpha; neutral AB inter-alpha (globulin) inhibitor	glucosidase, alpha; neutral AB inter-alpha (globulin) inhibitor H1 *	carbohydrate metabolic process
laminin, alpha 5	laminin, alpha 5 *	binding to cells via a high affinity receptor, laminin is thought to mediate the attachment, migration and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components
laminin, beta 1	laminin, beta 1 *	binding to cells via a high affinity receptor, laminin is thought to mediate the attachment, migration and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components
actin related protein 2/3 complex, subunit 4, 20kDa	actin related protein 2/3 complex, subunit 4, 20kDa *	actin-binding component of the Arp2/3 complex, involved in regulation of actin polymerization and together with nucleation-promoting factor (NPF) mediates the formation of branched actin networks

Paclitaxel-treated Caco-2	Palcitaxel-treated Caco-2 + Platelets	Protein Function
pregnancy-zone protein	pregnancy-zone protein *	able to inhibit all four classes of proteinases by a unique 'trapping' mechanism
clusterin	clusterin *	associated with programmed cell death (apoptosis)
peptidylprolyl isomerase B cyclophilin B	peptidylprolyl isomerase B cyclophilin B *	catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides
endoplasmic reticulum protein 29	endoplasmic reticulum protein 29 *	plays an important role in the processing of secretory proteins within the endoplasmic reticulum (ER)
serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1) *	binds specifically to collagen, stress response
protein disulfide isomerase family A, member 3	protein disulfide isomerase family A, member 3 *	catalyzes the rearrangement of -S-S- bonds in proteins

Tab. 3. Caco-2 cells were treated with paclitaxel (200 μg/ml) in the presence or absence of platelets (1.5 x 10^8/ml). Proteins marked * $P<0.05$ v/s respective controls.

The proteomics data were analysed by the protein function according to the database UniProt, www.uniprot.org

*Proteomics experiment performed by **Dr. Achim Treumann**, NEPAF Proteome Analysis Facility, Newcastle University, UK. The results of the statistical analysis carried out by **Dr. Howsun Jow**, Institute of Ageing and Health at Newcastle University, UK.*

Effects of Platelets on Cell Cycle in Caco-2 and 59M Cells in the Presence of Paclitaxel or 5-Fluorouracil

Fig. 32-37 show representative tracings taken from the flow cytometery of Caco-2 cell cycle, where P3 gate represents G_0/G_1 phase; P5 gate represents S phase and P4 gate represents G_2/M phase. To analyse in detail the data obtained from flow-cytometry I used ModFit LT™ software. The received histogram on the right of the figure shows the % of cells in particular phases such as G_0/G_1, S, G_2/M. The 5-fluorouracil treatment resulted in decreased number of cells in G_2/M phase.

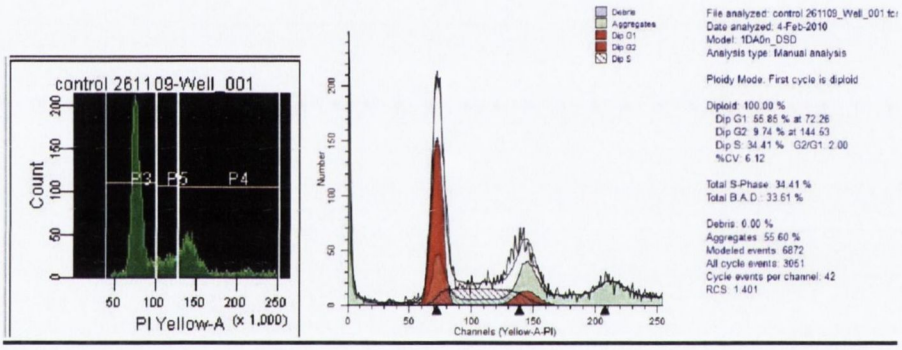


Fig. 32. Flow cytometry analysis of cell cycle untreated Caco-2 cells at 72 hrs (left hand side). The histogram on the right shows Caco-2 cell cycle obtained by ModFit LT™ software. Representative recordings of three similar experiments.

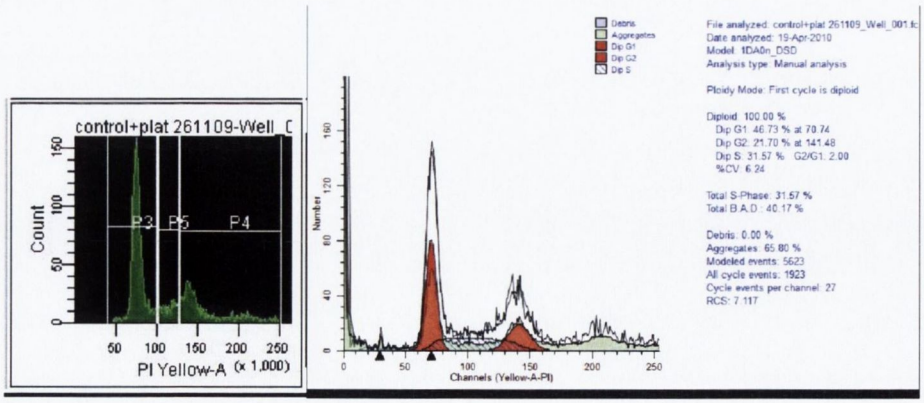


Fig. 33. Flow cytometry analysis of cell cycle untreated Caco-2 cells in the presence of platelets 1.5 x 10^8/ml (PLT) at 72 hrs (left hand side). The histogram on the right shows Caco-2 cell cycle obtained by ModFit LT™ software. Representative recordings of three similar experiments.

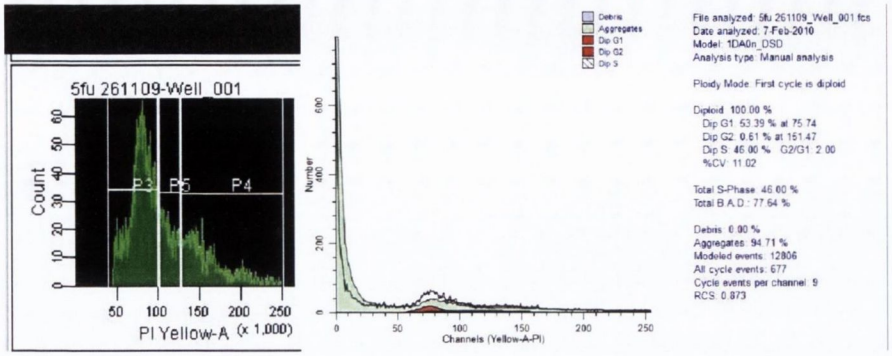


Fig. 34. Flow cytometry analysis of cell cycle Caco-2 cells treated with 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs (left hand side). The histogram on the right shows Caco-2 cell cycle obtained by ModFit LT™ software. Representative recordings of three similar experiments.

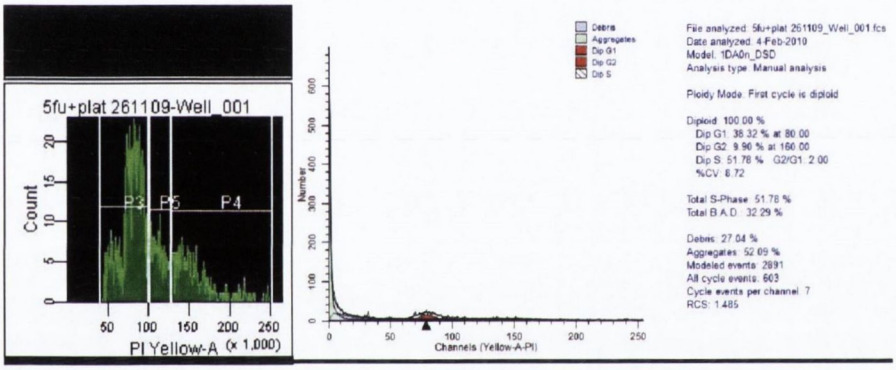


Fig. 35. Flow cytometry analysis of cell cycle Caco-2 cells treated with 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs in the presence of platelets 1.5 x 10^8/ml (PLT) (left hand side). The histogram on the right shows Caco-2 cell cycle obtained by ModFit LT™ software. Representative recordings of three similar experiments.

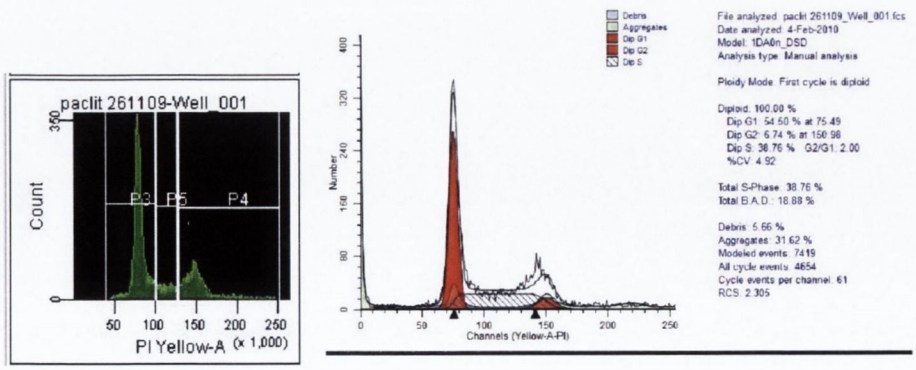


Fig. 36. Flow cytometry analysis of cell cycle Caco-2 cells treated with paclitaxel 200 µg/ml (PTX) at 72 hrs (left hand side). The histogram on the right shows Caco-2 cell cycle obtained by ModFit LT™ software. Representative recordings of three similar experiments.

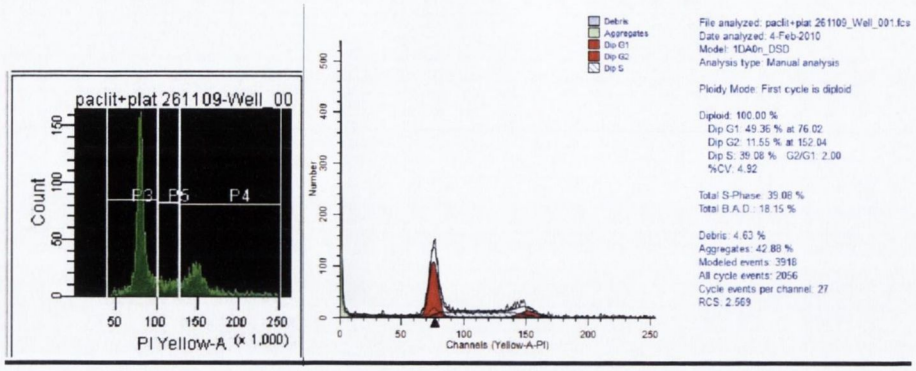


Fig. 37. Flow cytometry analysis of cell cycle Caco-2 cells treated with paclitaxel 200 μg/ml (PTX) at 72 hrs in the presence of platelets 1.5 x 10^8/ml (PLT) (left hand side). The histogram on the right shows Caco-2 cell cycle obtained by ModFit LT™ software. Representative recordings of three similar experiments.

Figures 38 and **39** show the statistical analysis of the effects of platelets on G_0/G_1, S and G_2/M phases in Caco-2 and 59M cell cycle in the presence or absence of treatments with 5-fluorouracil or paclitaxel. The presence of platelets resulted in decreased cell numbers in G_0/G_1 phase and increased number of cells in S, G_2/M phases.

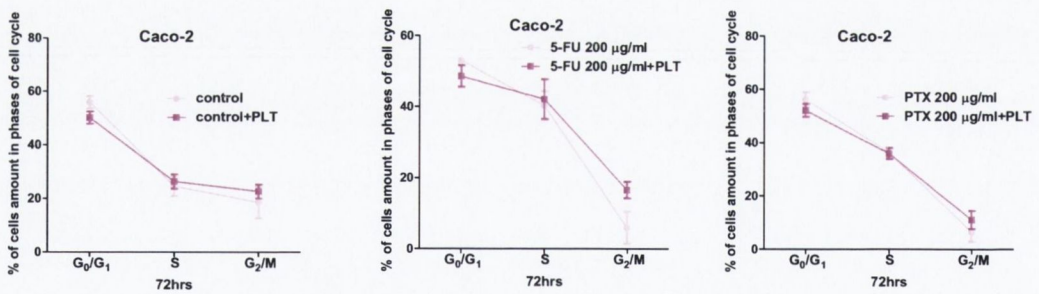
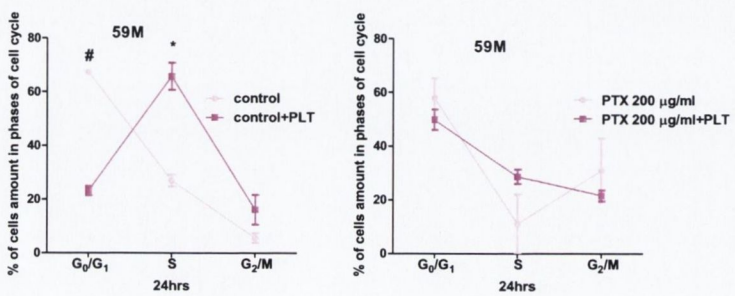


Fig. 38. There is a decreased number of cells in G_0/G_1 phase and increased number of cells in S, G_2/M phases in the presence of platelets. The curves show the effects of platelets at the concentration of 1.5 x 10^8/ml on G_0/G_1, S and G_2/M phases of Caco-2 untreated cell, Caco-2 cells treated with 5-fluorouracil 200 μg/ml and Caco-2 cells treated with paclitaxel 200 μg/ml at 72 hrs. Data are mean ± SD, n=3.



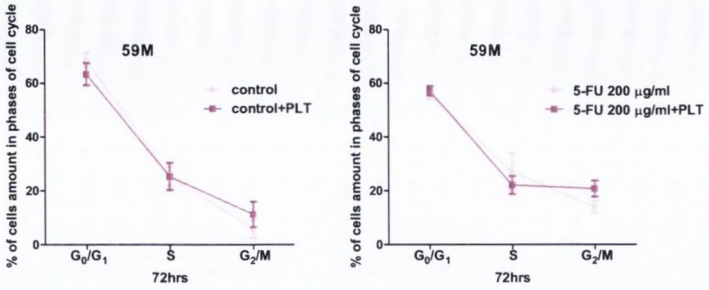


Fig. 39. There is decreased number of cells in G_0/G_1 phase and increased number of cells in S, G_2/M phases in the presence of platelets. The curves show the effects of platelets at the concentration of 1.5 x 10^8/ml on G_0/G_1, S and G_2/M phases of 59M untreated cell, 59M cells treated with 5-fluorouracil 200 µg/ml at 72 hrs and 59M cells treated with paclitaxel 200 µg/ml at 24 hrs. Data are mean ± SD, n=3. #P< 0.05 control G_0/G_1 v/s control+platelets G_0/G_1, *P<0.05 control S v/s control+platelets S.

Effects of Platelets on Cyclins A, B1, D1, E in 59M Cells in the Presence of Paclitaxel or 5-Fluorouracil in G_0/G_1, S and G_2/M Phases

I focused on analysing the levels of cyclins A, B1, D1 and E in 59M cells treated with paclitaxel or 5-fluorouracil in the presence or absence of platelets by using flow cytometry technique. Representative traces (**Fig. 40**) taken from the flow-cytometer show cyclin A in 59M cells in the presence or absence of paclitaxel (PTX, 200 µg/ml) or platelets at the concentration 1.5 x 10^8/ml at 24 hrs. The first quadrant (P2 gate) in pictures A, B, C and D represent the population of 59M cells. The next quadrant shows the expression amount of cyclin A particular in G_0/G_1, S, G_2/M phases. Gate P3 presents the negative amount of cyclin A during cell cycle, gate P7 shows the positive amount of cyclin A expressed in G_0/G_1 phase, gate P8 shows the positive amount of cyclin A expressed in S phase and gate P9 presents the positive amount of cyclin A expressed in G_2/M phase. The last quadrant shows the cell cycle histogram, where P6 gate represents G_0/G_1 phase; P4 gate represents S phase and P5 gate represents G_2/M phase.

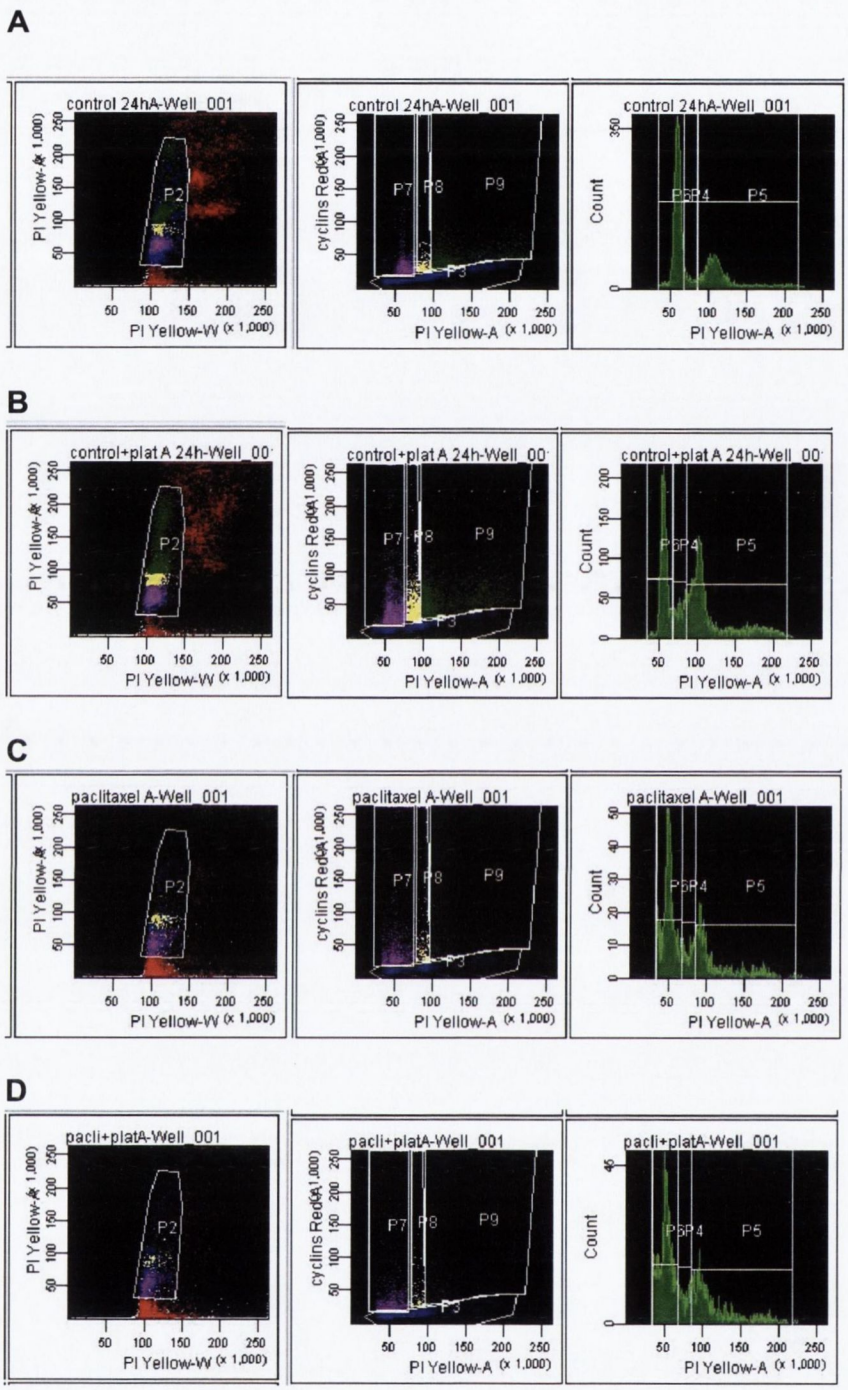


Fig. 40. Flow cytometry analysis of paclitaxel-treated 59M cells at 24 hrs. Representative recordings of 4 similar experiments showing the amounts of cyclin A during cell cycle experiments. A: Control incubates of 59M cells in the absence of platelets. B: Incubates of 59M cells with platelets. C: Incubates of 59M cells in the presence of paclitaxel (200 μg/ml). D: Incubates of 59M cells with platelets in the presence of paclitaxel (200 μg/ml). P2 gate: population of 59M cells, P3 gate: negative amount of cyclin A during cell cycle, P7 gate: positive amount of cyclin A expressed during G_0/G_1 phase, P8 gate: positive amount of cyclin A expressed during S phase, P9 gate: positive amount of cyclin A expressed during G_2/M phase. Last quadrant represents PI histogram of cell cycle, P6 gate: population of cells in G_0/G_1 phase, P4 gate: population of cells in S phase and P5 gate: population of cells in G_2/M phase.

Figures 41 and **42** show the statistical analysis of the effects of platelets on cyclin A expression in 59M cells. The effects of treatments on cyclin A expression in all cell cycle phases are shown in **figure 41**. **Figure 42** presents effects of treatments on cyclin A expression separately in G_0/G_1, S and G_2/M. The effect of platelets treatment resulted in upregulation of cyclin A expression in S and G_2/M phases, an effect inhibited by paclitaxel.

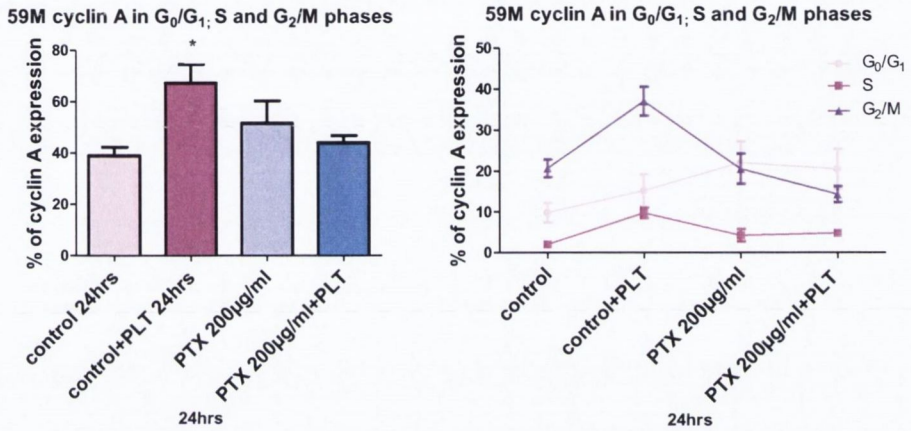


Fig. 41. Upregulation of cyclin A expression in untreated 59M cells by platelets. The bar graph on the left shows the % of cyclin A expression during the cell cycle in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and paclitaxel 200 µg/ml (PTX) at 24 hrs. The curves on the right present the changes of % cyclin A expression during the cell cycle and effects of platelets and paclitaxel on G_0/G_1, S and G_2/M phases of 59M. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets.

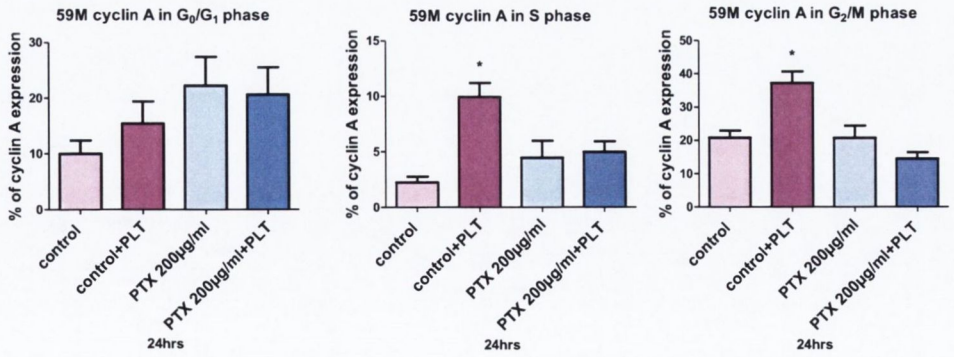


Fig. 42. Upregulation of cyclin A expression in untreated 59M cells by platelets. Each of bar graph shows the changes of % cyclin A expression in phases G_0/G_1, S and G_2/M of 59M cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and paclitaxel 200 µg/ml (PTX) at 24 hrs. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets.

Next, I analysed the effects of platelets on the expression of cyclin A in untreated and treated with 5-fluorouracil 59M cells at 72 hrs. **Figures 43** and **44** show the statistical analysis of the effects of platelets on cyclin A expression in 59M cells. The effects of treatments on cyclin A expression are shown in **figure 43**. **Figure 44** presents effects of treatments on cyclin A expression in cell cycle phases such as G_0/G_1, S and G_2/M. The platelet treatment resulted in upregulation of cyclin A expression in all phases of cell cycle progression. Similar effect was obtained by using 5-fluorouracil treatment in the presence of platelets, which resulted in upregulation of cyclin A expression particularly in G_0/G_1 and G_2/M phases.

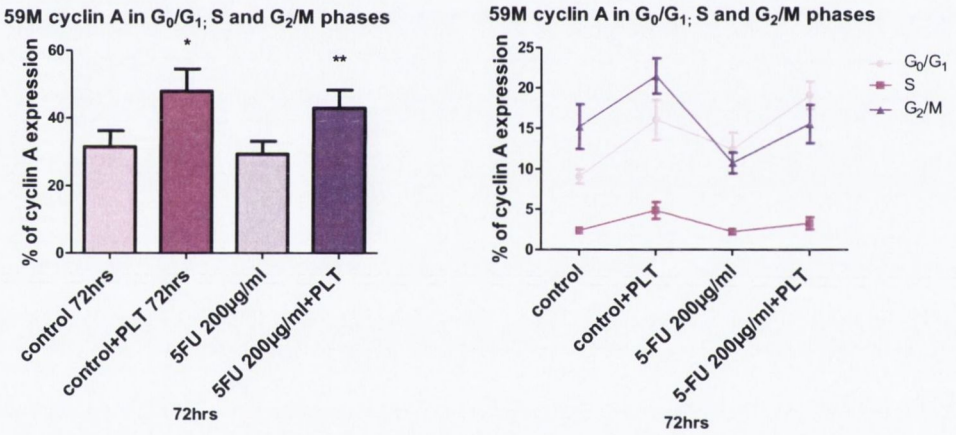


Fig. 43. Upregulation of cyclin A expression in untreated and treated with 5-fluorouracil 59M cells by platelets. The bar graph on the left shows the % of cyclin A expression during the cell cycle in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs. The curves on the right present the changes of % cyclin A expression during the cell cycle and effects of platelets and 5-fluorouracil on G_0/G_1, S and G_2/M phases of 59M. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets, **P<0.05 5-FU v/s 5-FU+platelets.

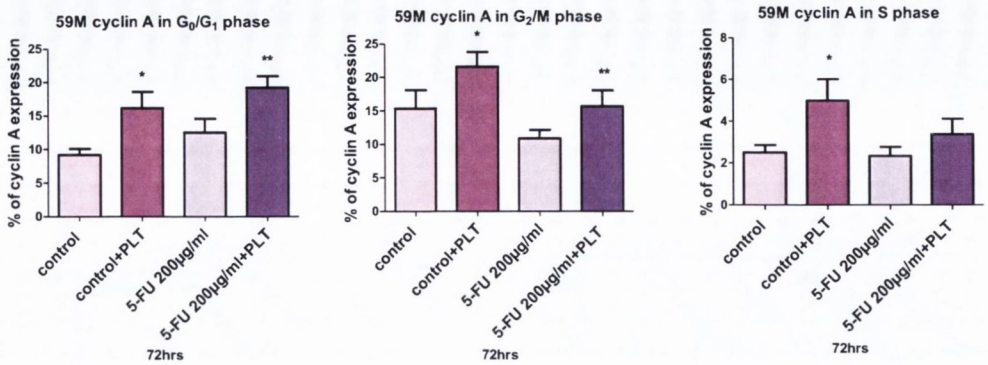


Fig. 44. Upregulation of cyclin A expression in untreated and treated with 5-fluorouracil 59M cells by platelets. Each of bar graph show the changes of % cyclin A expression in the particular phases G_0/G_1, S and G_2/M of 59M cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets. **P<0.05 5-FU v/s 5-FU+platelets.

I also analysed the effects of platelets on the expression of cyclin B1 in untreated and treated with paclitaxel 59M cells at 24 hrs. **Figures 45** and **46** show the statistical analysis of the effects of platelets on cyclin B1 expression in 59M cells. The effects of treatments on cyclin B1 expression are shown in **figure 45**. **Figure 46** presents effects of treatments on cyclin B1 expression in cell cycle phases such as G_0/G_1, S and G_2/M. The effect of platelets resulted in upregulation of cyclin B1 expression in G_2/M phases, an effect inhibited by paclitaxel.

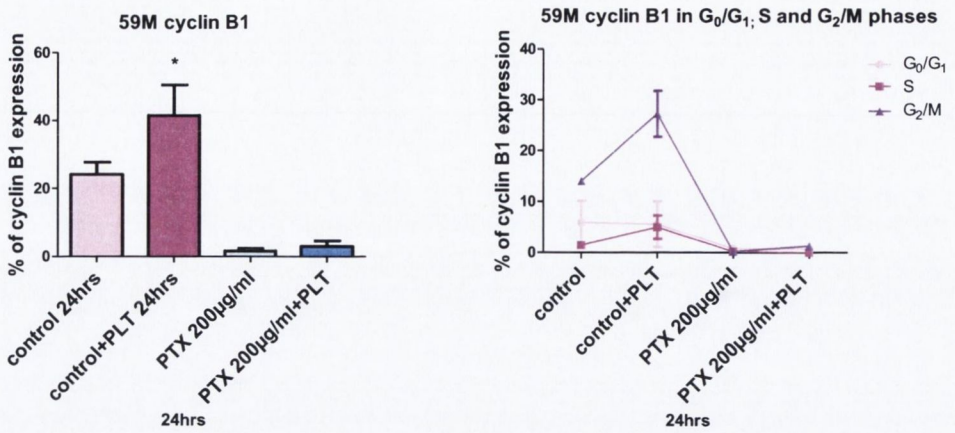


Fig. 45. Upregulation of cyclin B1 expression in untreated 59M cells by platelets. The bar graph on the left shows the % of cyclin B1 expression during the cell cycle in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and paclitaxel 200 µg/ml (PTX) at 24 hrs. The curves on the right present the changes of % cyclin B1 expression during the cell cycle and effects of platelets and paclitaxel on G_0/G_1, S and G_2/M phases of 59M. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets.

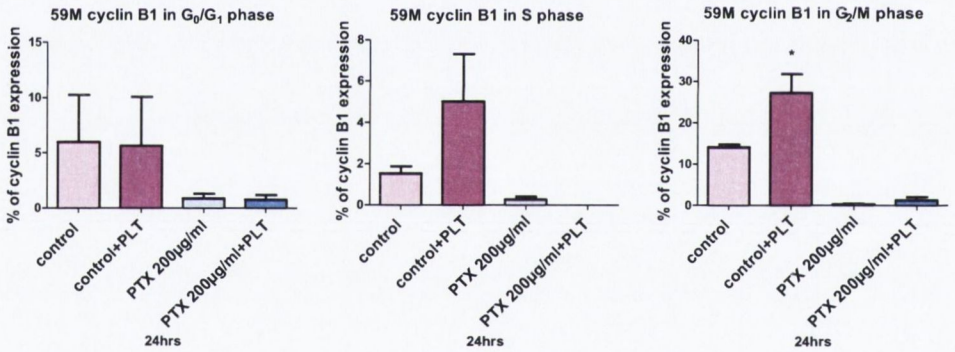


Fig. 46. Upregulation of cyclin A expression in untreated 59M cells by platelets. Each of bar graph shows the changes of % cyclin B1 expression in the particular phases G_0/G_1, S and G_2/M of 59M cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and paclitaxel 200 µg/ml (PTX) at 24 hrs. Data are mean ± SD, n=3. *P< 0.05 untreated cells v/s cells + platelets.

The next analysis was focused on the effects of platelets on the expression of cyclin B1 in untreated and treated with 5-fluorouracil 59M cells at 72 hrs. **Figures 47** and **48** show the statistical analysis of the effects of platelets on cyclin B1 expression in 59M cells. The effects of treatments on cyclin B1 expression are shown in **figure 47**. **Figure 48** presents effects of treatments on cyclin B1 expression in cell cycle phases such as G_0/G_1, S and G_2/M. The

platelet treatment resulted in upregulation of cyclin B1 expression. Similar effect was obtained with 5-fluorouracil in the presence of platelets, which resulted in upregulation of cyclin B1 expression particularly in G_0/G_1 and S phases.

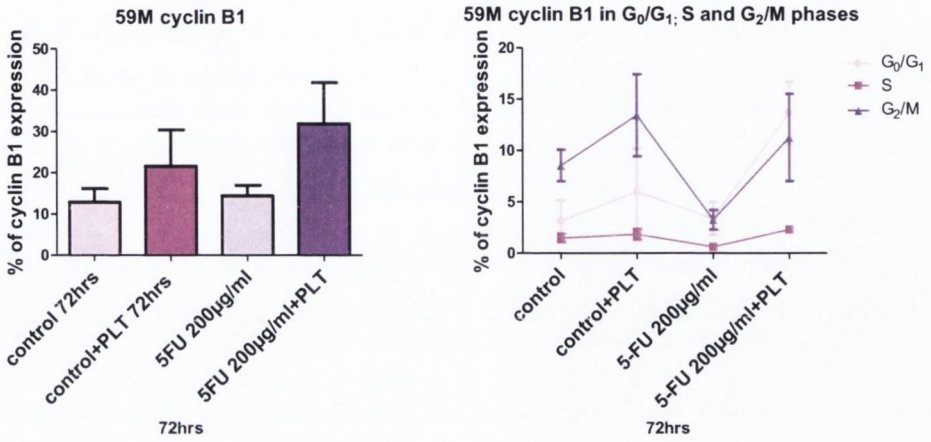


Fig. 47. Upregulation of cyclin B1 expression in untreated and treated with 5-fluorouracil 59M cells by platelets. The bar graph on the left shows the % of cyclin B1 expression during the cell cycle in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs. The curves on the right present the changes of % cyclin B1 expression during the cell cycle and effects of platelets and 5-fluorouracil on G_0/G_1, S and G_2/M phases of 59M. Data are mean ± SD, n=3.

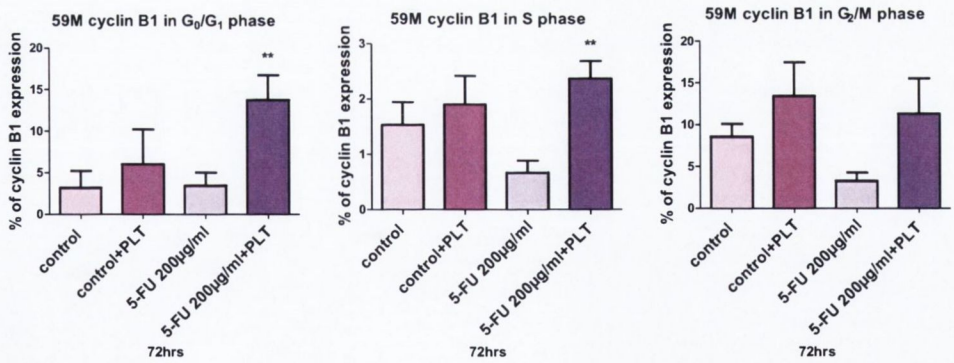


Fig. 48. Upregulation of cyclin B1 expression in untreated and treated with 5-fluorouracil 59M cells by platelets. Each of bar graph shows the changes of % cyclin B1 expression in the particular phases G_0/G_1, S and G_2/M of 59M cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs. Data are mean ± SD, n=3. **P<0.05 5-FU v/s 5-FU+platelets.

I analysed also the effects of platelets on the expression of cyclin D1 in untreated and treated with paclitaxel 59M cells at 24 hrs. **Figures 49** and **50**

show the statistical analysis of the effects of platelets on cyclin D1 expression in 59M cells. The effects of treatments on cyclin D1 expression are shown in **figure 49**. **Figure 50** presents effects of treatments on cyclin D1 expression in cell cycle phases such as G_0/G_1, S and G_2/M. The platelets treatment resulted in upregulation of cyclin D1 expression in S and G_2/M phases, and this effect was inhibited by paclitaxel.

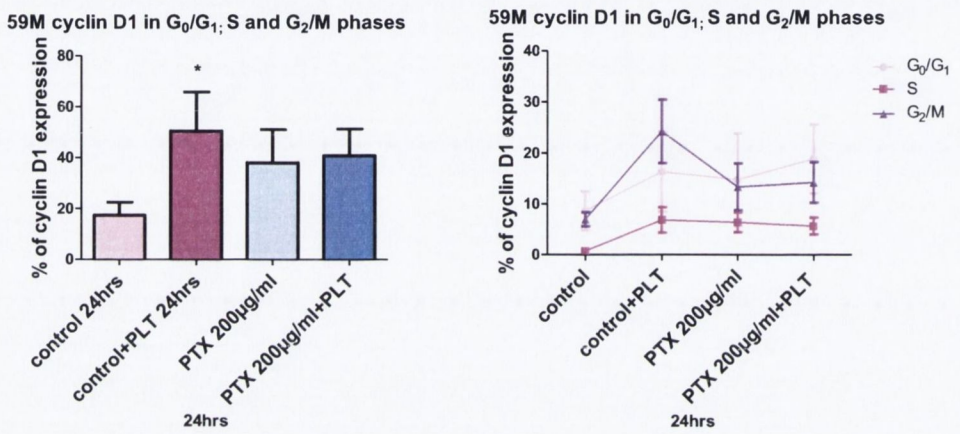


Fig. 49. Upregulation of cyclin D1 expression in untreated 59M cells by platelets. The bar graph on the left shows the % of cyclin D1 expression during the cell cycle in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and paclitaxel 200 µg/ml (PTX) at 24 hrs. The curves on the right present the changes of % cyclin D1 expression during the cell cycle and effects of platelets and paclitaxel on G_0/G_1, S and G_2/M phases of 59M. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets.

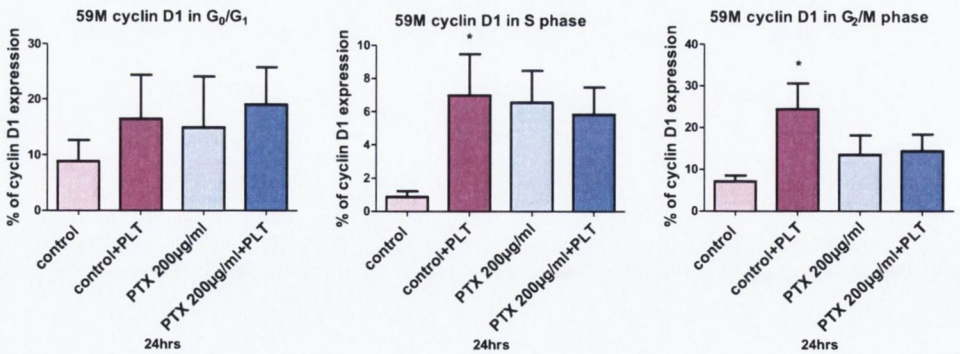


Fig. 50. Upregulation of cyclin D1 expression in untreated 59M cells by platelets. Each of bar graph shows the changes of % cyclin D1 expression in the particular phases G_0/G_1, S and G_2/M of 59M cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and paclitaxel 200 µg/ml (PTX) at 24 hrs. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets.

Next, I analysed the effects of platelets on the expression of cyclin D1 in untreated and treated with 5-fluorouracil 59M cells at 72 hrs. **Figures 51** and **52** show the statistical analysis of the effects of platelets on cyclin D1 expression in 59M cells. The effects of treatments on cyclin D1 expression are shown in **figure 51**. **Figure 52** presents effects of treatments on cyclin D1 expression in cell cycle phases such as G_0/G_1, S and G_2/M. The effect of platelets resulted in upregulation of cyclin D1 expression particularly in S and G_2/M phases. Similar effect was obtained by using 5-fluorouracil treatment in the presence of platelets, which resulted in upregulation of cyclin D1 expression particularly in S phase.

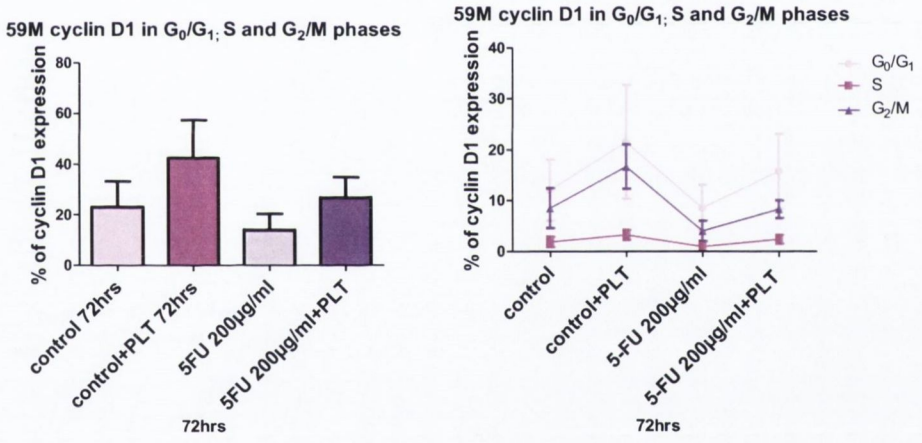


Fig. 51. Upregulation of cyclin D1 expression in untreated and treated with 5-fluorouracil 59M cells by platelets. The bar graph on the left shows the % of cyclin D1 expression during the cell cycle in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs. The curves on the right present the changes of % cyclin D1 expression during the cell cycle and effects of platelets and 5-fluorouracil on G_0/G_1, S and G_2/M phases of 59M. Data are mean ± SD, n=3.

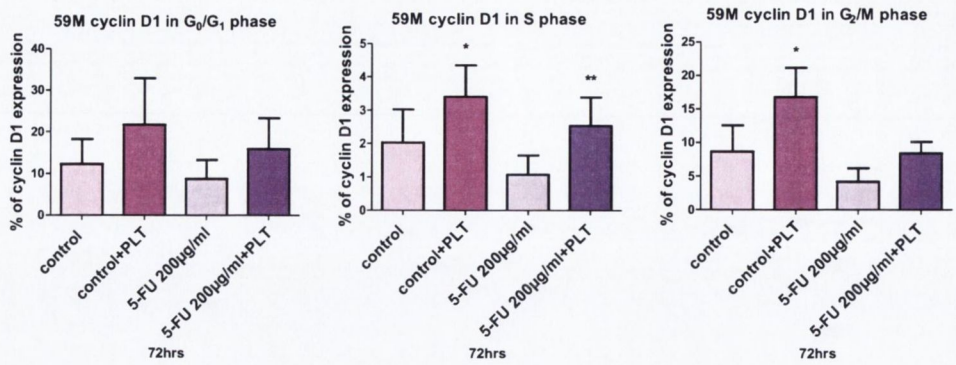


Fig. 52. Upregulation of cyclin D1 expression in untreated and treated with 5-fluorouracil 59M cells by platelets. Each of bar graph shows the changes of % cyclin D1 expression in the particular phases G_0/G_1, S and G_2/M of 59M cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs. Data are mean ± SD, n=3. P*<0.05 untreated cells v/s cells+platelets, **P<0.05 5-FU v/s 5-FU+platelets.

I analysed the effects of platelets on the expression of cyclin E in untreated and treated with paclitaxel 59M cells at 24 hrs. **Figures 53** and **54** show the statistical analysis of the effects of platelets on cyclin E expression in 59M cells. The effects of treatments on cyclin E expression are shown in **figure 53**. **Figure 54** presents effects of treatments on cyclin E expression in cell cycle phases such as G_0/G_1, S and G_2/M. The platelets treatment resulted in upregulation of cyclin E expression in all the phases of cell cycle progression, an effect inhibited by paclitaxel.

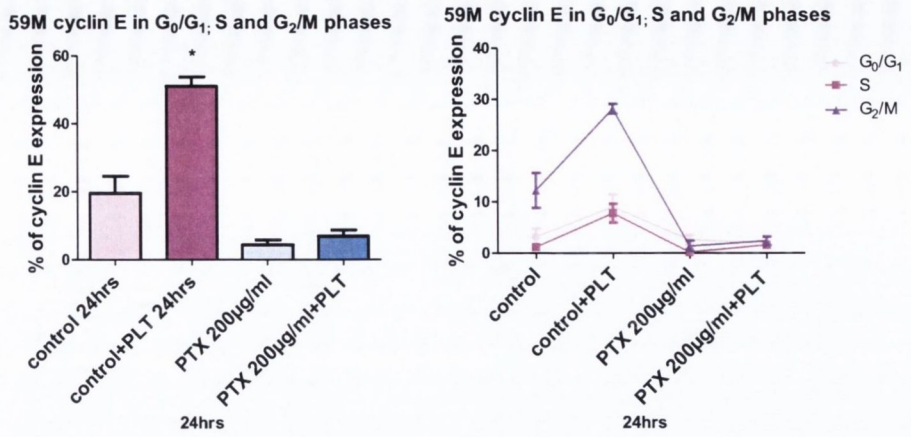


Fig. 53. Upregulation of cyclin E expression in untreated 59M cells by platelets. The bar graph on the left shows the % of cyclin E expression during the cell cycle in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and paclitaxel 200 µg/ml (PTX) at 24 hrs. The curves on the right present the changes of % cyclin E expression during the cell cycle and effects of platelets and paclitaxel on G_0/G_1, S and G_2/M phases of 59M. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets.

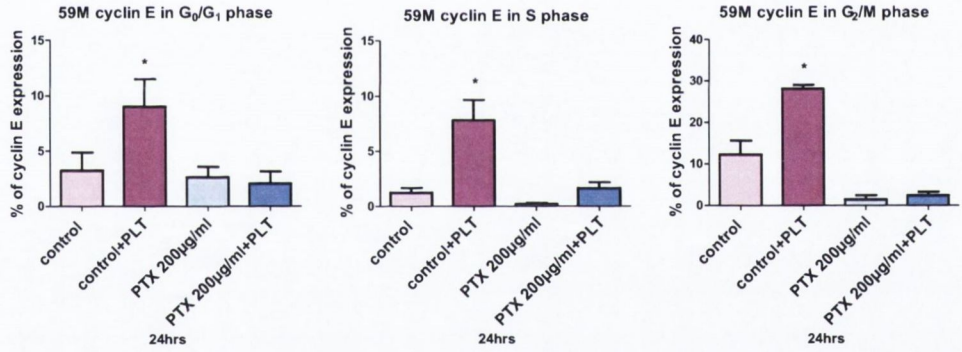


Fig. 54. Upregulation of cyclin E expression in untreated 59M cells by platelets. Each of bar graph shows the changes of % cyclin E expression in the particular phases G_0/G_1, S and G_2/M of 59M cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and paclitaxel 200 µg/ml (PTX) at 24 hrs. Data are mean ± SD, n=4. *P< 0.05 untreated cells v/s cells + platelets.

Finally, I analysed the effects of platelets on the expression of cyclin E in untreated and treated with 5-fluorouracil 59M cells at 72 hrs. **Figures 55** and **56** show the statistical analysis of the effects of platelets on cyclin E expression in 59M cells. The effects of treatments on cyclin E expression are shown in **figure 55**. **Figure 56** presents effects of treatments on cyclin E expression in cell cycle phases such as G_0/G_1, S and G_2/M. The effect of platelets resulted in

upregulation of cyclin E expression. Similar effect was obtained by using 5-fluorouracil treatment in the presence of platelets, which resulted in upregulation of cyclin E expression.

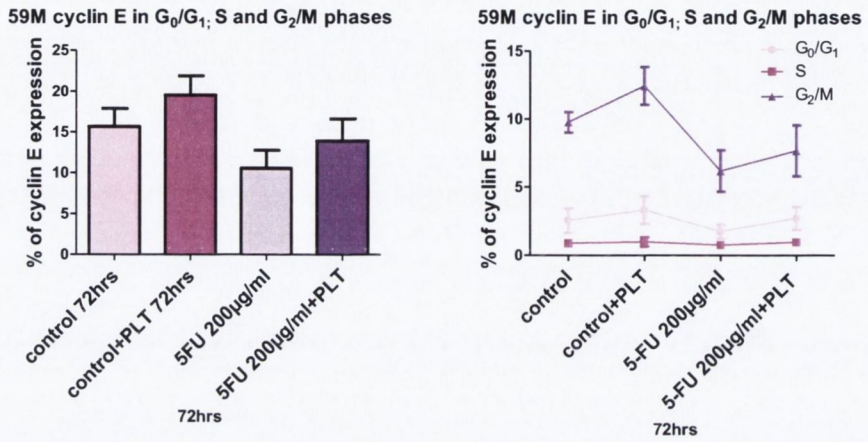


Fig. 55. Upregulation of cyclin E expression in untreated and treated with 5-fluorouracil 59M cells by platelets. The bar graph on the left shows the % of cyclin E expression during the cell cycle in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs. The curves on the right present the changes of % cyclin E expression during the cell cycle and effects of platelets and 5-fluorouracil on G_0/G_1, S and G_2/M phases of 59M. Data are mean ± SD, n=3.

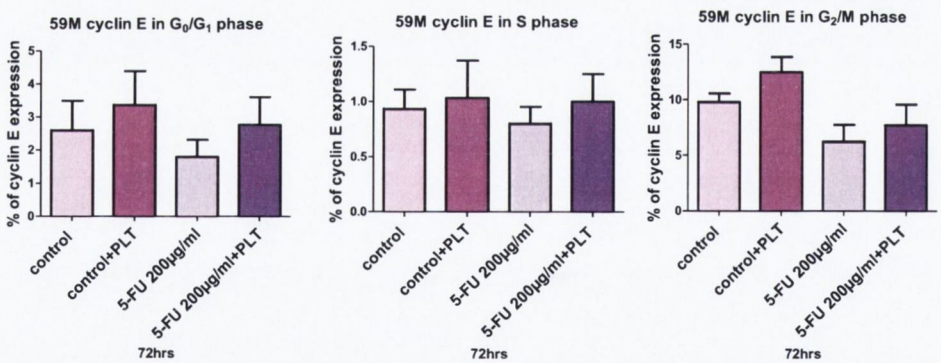


Fig. 56. Upregulation of cyclin E expression in untreated and treated with 5-fluorouracil 59M cells by platelets. Each of bar graph shows the changes of % cyclin E expression in the particular phases G_0/G_1, S and G_2/M of 59M cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT) and 5-fluorouracil 200 µg/ml (5-FU) at 72 hrs. Data are mean ± SD, n=3.

Effects of Platelets on DNA Damage Repair Proteins in Caco-2 and 59M Cells in the Presence of Paclitaxel or 5-Fluorouracil

To study the effects of platelets on the active form and total amount of BRCA1 protein I used Western Blot technique. **Figure 57** shows the representative insets of blots (phospho-BRCA1and control tubulin) and the statistical analysis of phospho-BRCA1. Platelets resulted in up-regulation of the BRCA1 protein in untreated and treated with paclitaxel (PTX) or 5-fluorouracil (5FU) 59M and Caco-2 cells.

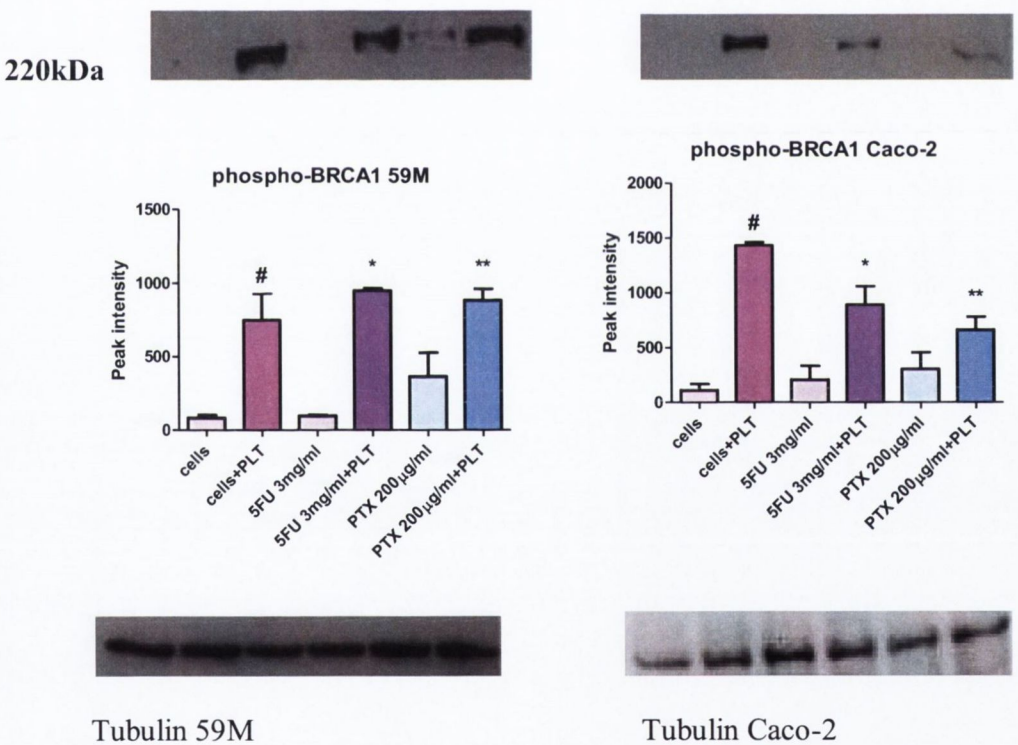


Fig. 57. Upregulation of phospho- and total BRCA1 proteins amount in untreated and treated with 5-fluorouracil or paclitaxel 59M and Caco-2 cells by platelets. The amount of protein phospho-BRCA1, total BRCA1 and tubulin in 59M and Caco-2 cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT), 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 µg/ml (PTX) at 1 hour. Data are mean ± SD, n=4.
#P<0.05 untreated cells v/s cells+platelets, *P<0.05 5-FU v/s 5-FU+platelets, **P<0.05 PTX v/s PTX+platelets.

Next, I analysed the effects of platelets on the active form and total amount of checkpoint 1 protein by Western Blot technique. **Figure 58** shows the statistical analysis of phospho-Chk1. The effect of platelets resulted in up-regulation of the Chk1 protein in untreated and treated with paclitaxel (PTX) or 5-fluorouracil (5FU) 59M and Caco-2 cells.

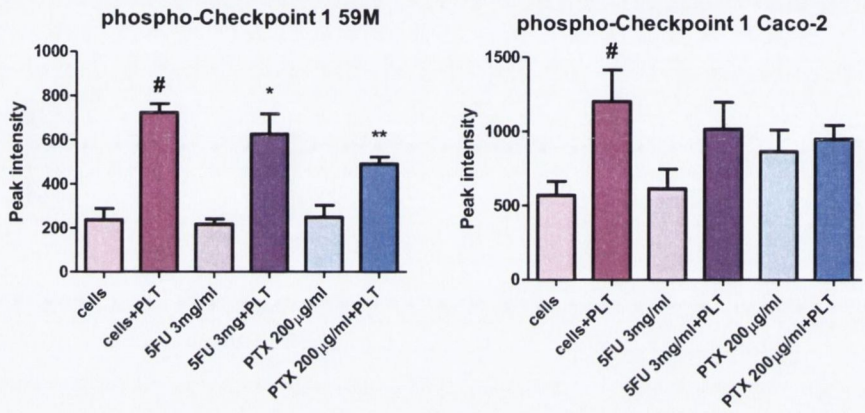


Fig. 58. Upregulation of phospho- and total Chk1 proteins amount in untreated and treated with 5-fluorouracil or paclitaxel 59M and Caco-2 cells by platelets. The amount of protein phospho-checkpoint 1, total checkpoint1 in 59M and Caco-2 cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT), 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 µg/ml (PTX) at 1 hour. Data are mean ± SD, n=4.
#P<0.05 untreated cells v/s cells+platelets, *P<0.05 5-FU v/s 5-FU+platelets, **P<0.05 PTX v/s PTX+platelets.

I analysed the effects of platelets on the active form and total amount of Mre11 protein by Western Blot technique. **Figure 59** shows the statistical analysis of phospho-Mre11. The platelets treatment resulted in up-regulation of the Mre11 protein in untreated and treated with paclitaxel (PTX) or 5-fluorouracil (5FU) 59M and Caco-2 cells.

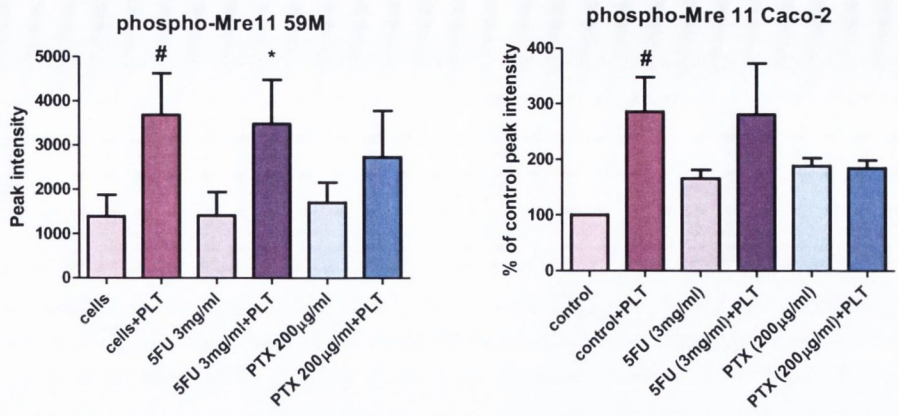


Fig. 59. Upregulation of phospho- and total Mre11 proteins amount in untreated and treated with 5-fluorouracil or paclitaxel 59M and Caco-2 cells by platelets.The amount of protein phospho-Mre11, total Mre11 and tubulin in 59M and Caco-2 cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT), 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 µg/ml (PTX) at 1 hour. Data are mean ± SD, n=4. #P< 0.05 untreated cells v/s cells+platelets, *P<0.05 5-FU v/s 5-FU+platelets.

Next, I analysed the effects of platelets on the active form and total amount of p95/Nbs1 protein by Western Blot technique. **Figure 60** shows the statistical analysis of phospho-p95/Nbs1. The effect of platelets resulted in up-regulation of the p95/Nbs1 protein in untreated and treated with paclitaxel (PTX) or 5-fluorouracil (5FU) 59M and Caco-2 cells.

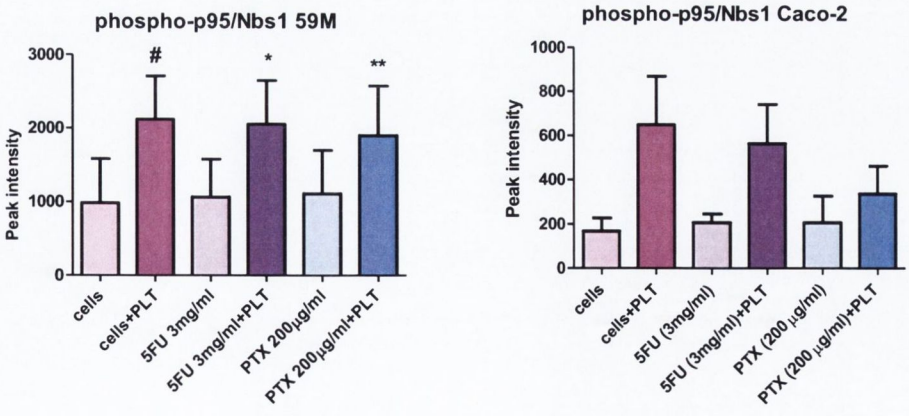


Fig. 60. Upregulation of phospho- and total p-95/Nbs1 proteins amount in untreated and treated with 5-fluorouracil or paclitaxel 59M and Caco-2 cells by platelets. The amount of protein phospho-p95/Nbs1, total p95/Nbs1 in 59M and Caco-2 cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT), 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 µg/ml (PTX) at 1 hour. Data are mean ± SD, n=4. #P<0.05 untreated cells v/s cells+platelets, *P<0.05 5-FU v/s 5-FU+platelets, **P<0.05 PTX v/s PTX+platelets.

Finally, I analysed the effects of platelets on the active form of H2A.X protein by Western Blot technique. **Figure 61** shows the statistical analysis of the data. The platelets treatment resulted in down-regulation of the H2A.X protein in untreated and treated with paclitaxel (PTX) or 5-fluorouracil (5FU) 59M and Caco-2 cells.

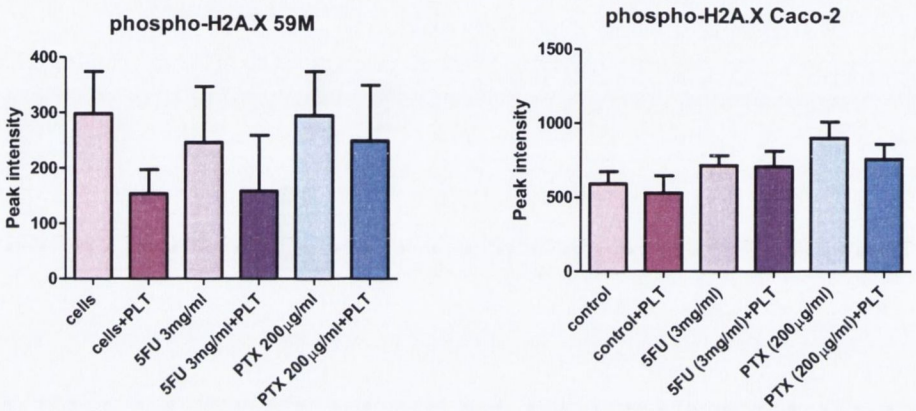


Fig. 61. Downregulation of phospho-H2A.X protein amount in untreated and treated with 5-fluorouracil or paclitaxel 59M and Caco-2 cells by platelets. The amount of protein phospho-H2A.X in 59M and Caco-2 cells in the presence or absence of platelets 1.5 x 10^8/ml (PLT), 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 μg/ml (PTX) at 1 hour.

Effects of Platelets on Mitogen-Activated Protein (MAP) Kinase Pathways in Caco-2 and 59M Cells in the Presence of Paclitaxel or 5-Fluorouracil

To study the effects of platelets on the active forms of p38 and p42/44 MAP kinase pathways proteins I used Western Blot technique. **Figure 62** shows the representative insets of blots (phospho-p38, phospho-p42/44) and the statistical analysis of phospho-p38 and phospho-p42/44. The platelet treatment resulted in up-regulation of the phospho-p38 in untreated and treated with paclitaxel (PTX) or 5-fluorouracil (5FU) 59M cells. Platelets resulted also in up-regulation of phospho-p42/44 protein MAPK pathway in untreated 59M cells.

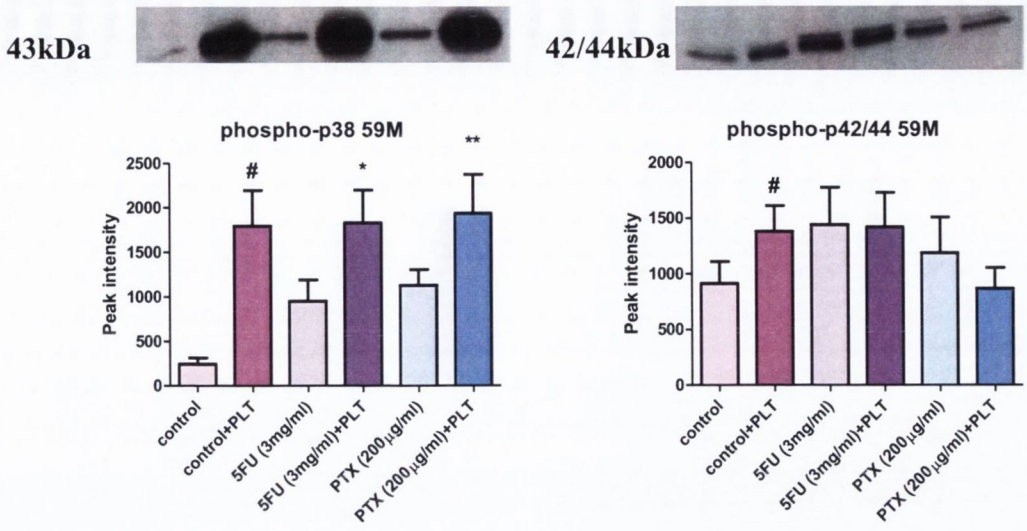


Fig. 62. Upregulation of phospho-p38 and phospho-p42/44 proteins amount by treatment with 5-fluorouracil or paclitaxel by platelets in 59M cells. The amount of protein phospho-p38 and phospho-p42/44 in 59M cells in the presence of platelets, 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 µg/ml (PTX) at 1 hour. Data are mean ± SD, n=4. #P< 0.05 untreated cells v/s cells+platelets, *P<0.05 5-FU v/s 5-FU+platelets, **P<0.05 PTX v/s PTX+platelets.

Next, I analysed the effects of platelets on the active forms of p46 JNK and p54 JNK MAP kinase pathways proteins by Western Blot technique. **Figure 63** shows the statistical analysis of phospho-p46 JNK and phospho-p54 JNK. The platelet treatment resulted in up-regulation of the phospho-p54 JNK in untreated and treated with 5-fluorouracil (5FU) 59M cells. The effect of platelets resulted also in up-regulation of phospho-p46 JNK protein MAPK pathway in untreated 59M cells.

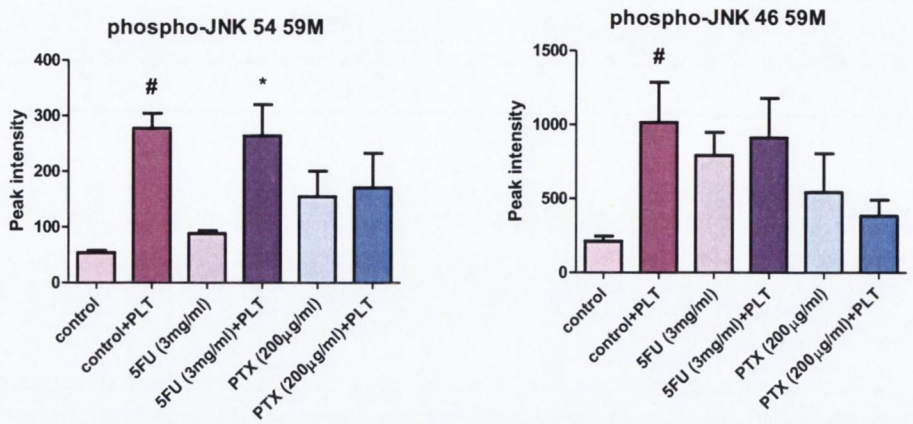


Fig. 63. Upregulation of phospho-p54 JNK and phospho-p46 JNK proteins amount by treatment with 5-fluorouracil or paclitaxel by platelets in 59M cells. The amount of protein phospho-p54 JNK and phospho-p46 JNK in 59M cells in the presence of platelets, 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 µg/ml (PTX) at 1 hour. Data are mean ± SD, n=4. #P< 0.05 untreated cells v/s cells+platelets, *P<0.05 5-FU v/s 5-FU+platelets.

Next, I analysed the effects of platelets on the active forms of p38 and p42/44 MAP kinase pathways proteins by Western Blot technique. **Figure 64** shows the statistical analysis of phospho-p38 and phospho-p42/44. The platelet treatment resulted in up-regulation of the phospho-p38 in untreated and treated with paclitaxel (PTX) or 5-fluorouracil (5FU) Caco-2 cells. Platelets resulted also in up-regulation of phospho-p42/44 protein MAPK pathway in untreated Caco-2 cells. Similar results were obtained by using 59M cells.

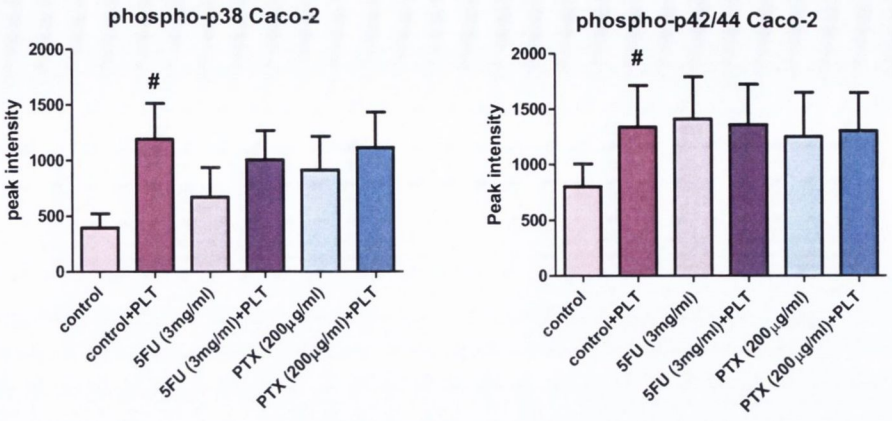


Fig. 64. Upregulation of phospho-p38 and phospho-p42/44 proteins amount by treatment with 5-fluorouracil or paclitaxel by platelets in Caco-2 cells. The amount of protein phospho-p38 and phospho-p42/44 in Caco-2 cells in the presence of platelets, 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 µg/ml (PTX) at 1 hour. Data are mean ± SD, n=4. Data are mean ± SD, n=4. #P< 0.05 untreated cells v/s cells+platelets.

Finally, I analysed the effects of platelets on the active forms of p46 JNK and p54 JNK MAP kinase pathways proteins by Western Blot. **Figure 65** shows t the statistical analysis of phospho-p46 JNK and phospho-p54 JNK. The effect of platelets resulted in up-regulation of the phospho-p54 JNK in untreated and treated with 5-fluorouracil (5FU) or paclitaxel (PTX) Caco-2 cells. Platelets resulted also in up-regulation of phospho-p46 JNK protein MAPK pathway in untreated Caco-2 cells.

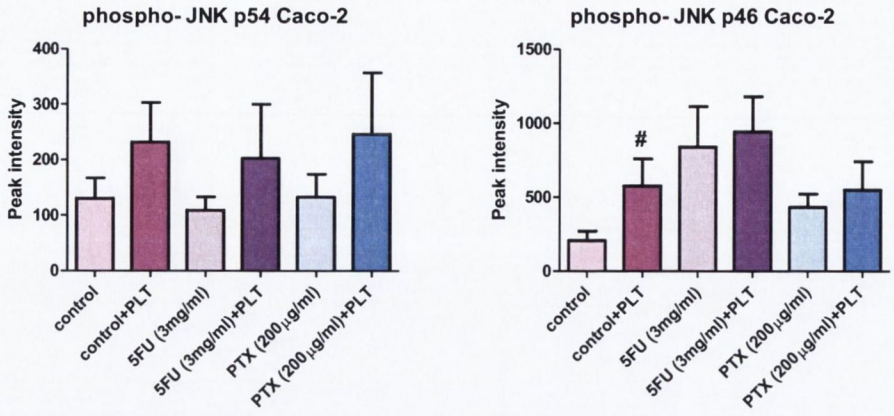


Fig. 65. Upregulation of phospho-p54 JNK and phospho-p46 JNK proteins amount by treatment with 5-fluorouracil or paclitaxel by platelets in Caco-2 cells. The amount of protein phospho-p54 JNK and phospho-p46 JNK in Caco-2 cells in the presence of platelets, 5-fluorouracil 3 mg/ml (5FU) and paclitaxel 200 µg/ml (PTX) at 1 hour. Data are mean ± SD, n=4. #P< 0.05 untreated cells v/s cells+platelets.

Effects of Platelets on Telomerase Activity in Caco-2 Cells in the Presence of 5-Fluorouracil

To analyse the telomerase activity in Caco-2 cells I have used TeloTAGGG Telomerase PCR ELISA^{PLUS} kit from Roche. This is two-steps assay: elongation/amplification (TRAP assay) and detection by ELISA. As shown in **Fig. 66** platelets did not significantly affect (p>0.05) the activity of telomerase in untreated cells and cells treated with 5-fluorouracil.

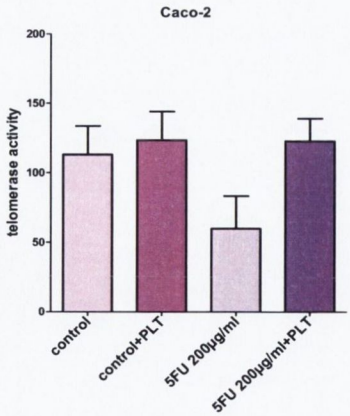


Fig. 66. Telomerase activity in Caco-2 cells in the presence of platelets 1.5 x 10^8/ml (PLT). Caco-2 cells treated with 5-fluorouracil 200 µg/ml (5FU) at 24 hrs. Data are mean ± SD, n=3, untreated cells v/s cells+platelets

Phase Contrast Microscopy

Phase-contrast microscopy was performed to study platelet effect on Caco-2 and 59M cells in the presence or absence of chemotherapeutic treatments such as paclitaxel 200 µg/ml (PTX) or 5-fluorouracil 200 µg/ml (5FU). Platelets appear to improve the morphology of cancer in the presence of chemotherapeutic agents. (**Fig. 67A-67N**).

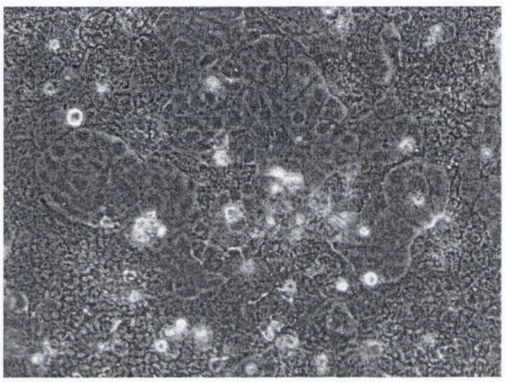


Fig. 67A. Caco-2 untreated cells 72 hrs, 20x.

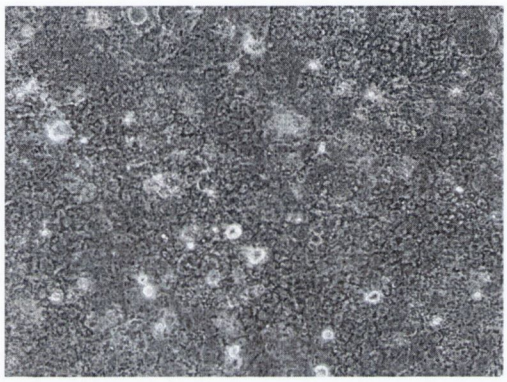


Fig. 67B. Caco2 untreated cells+platelets 72 hrs, 20x.

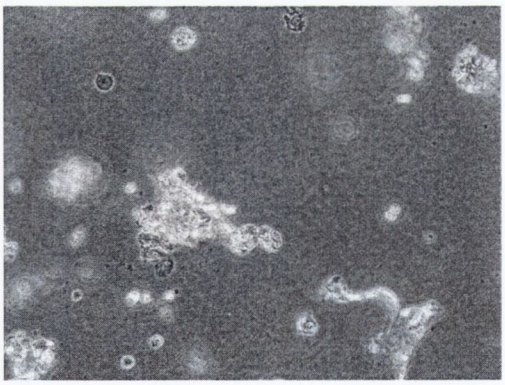


Fig. 67C. Caco-2 PTX 72 hrs, 20x.

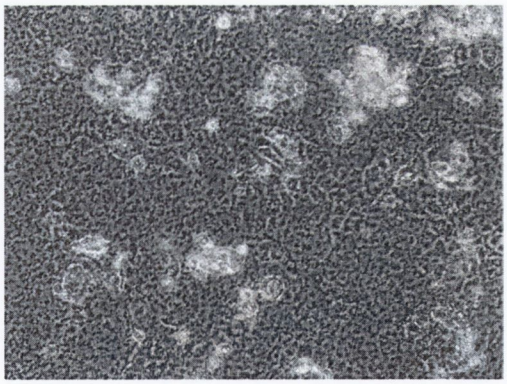


Fig. 67D. Caco-2 PTX + platelets 72 hrs, 20x.

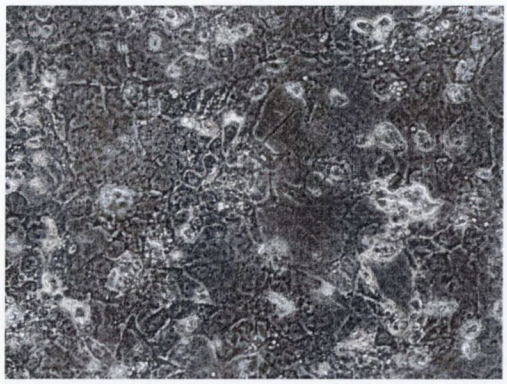


Fig. 67E. Caco2 5-FU 72 hrs, 20x.

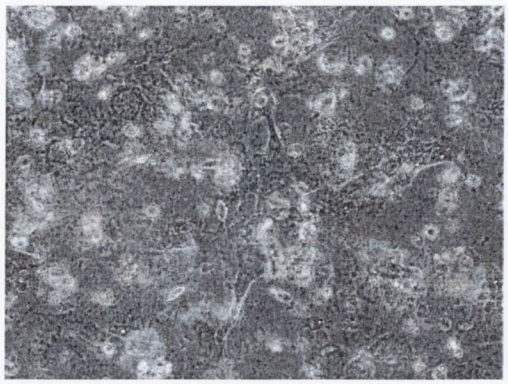


Fig. 67F. Caco2 5-FU + platelets 72 hrs, 20x.

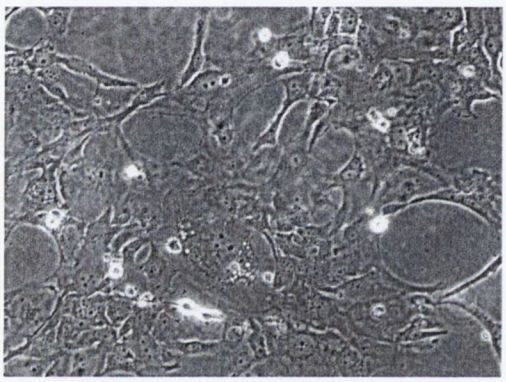


Fig. 67G. 59M untreated cells 24 hrs, 20x.

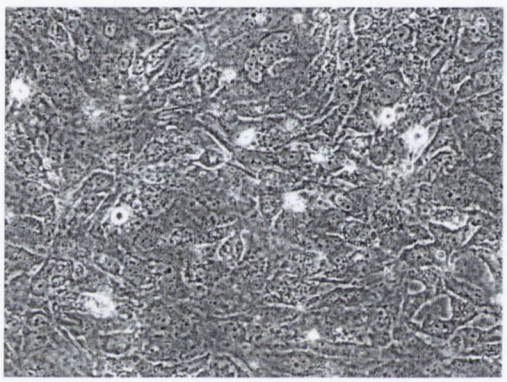


Fig. 67H. 59M untreated cells+platelets 24 hrs, 20x.

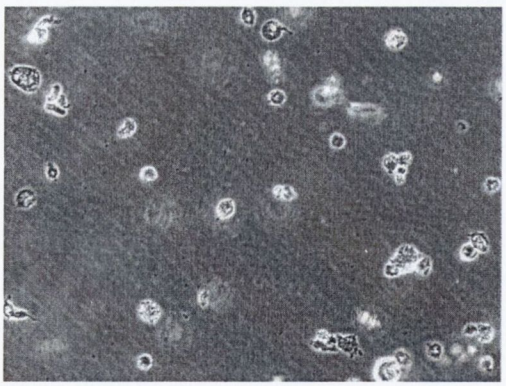


Fig. 67I. 59M PTX 24 hrs, 20x.

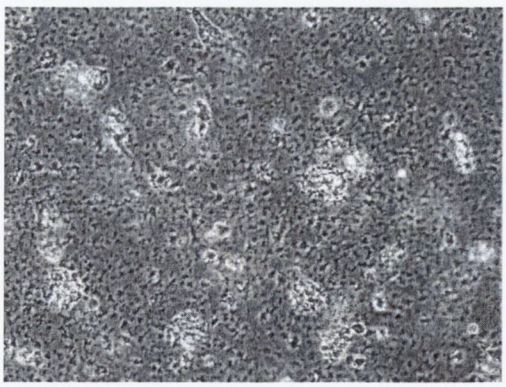


Fig. 67J. 59M PTX + platelets 24 hrs, 20x.

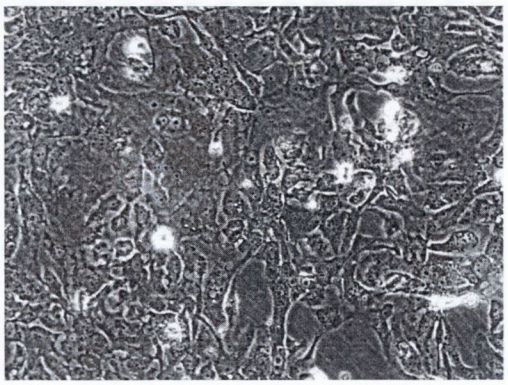


Fig. 67K. 59M untreated cells 72 hrs, 20x.

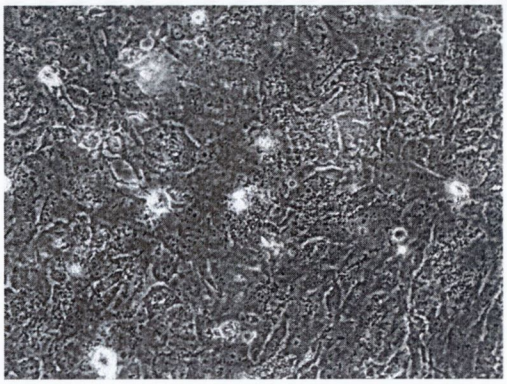


Fig. 66L. 59M untreated cells+platelets 72 hrs, 20x.

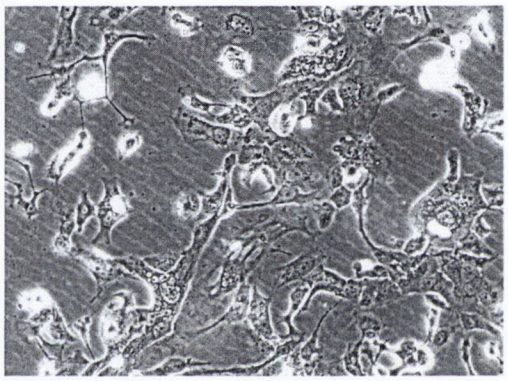


Fig. 67M. 59M 5-FU 72 hrs, 20x.

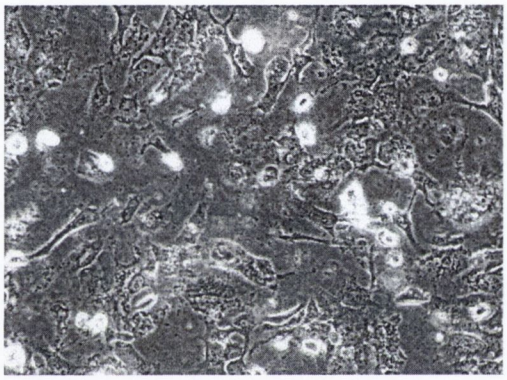


Fig. 67N. 59M 5-FU + platelets 72 hrs, 20x.

DISCUSSION

Key Novel Findings

I have shown that platelets increase survival of human ovarian and colonic cancer cells when challenged with anticancer agents: paclitaxel and 5-fluorouracil. Mechanisms involved in this effect of platelets are modulation of cancer cell apoptosis, cell cycle, DNA damage repair and MAP kinase activity.

Apoptosis

I have provided a proof-of-principle for the concept that platelets modulate paclitaxel- and 5-fluorouracil-induced cancer cell apoptosis. In the experimental model used, incubation of two human cancer cell lines (Caco-2 colon adenocarcinoma cells and 59M ovarian tumour epithelial cells) with two cancer chemotherapeutic agents with different mechanism of action i.e. paclitaxel (mitotic spindle poison) and 5-fluorouracil (pyrimidine antagonist) led to apoptosis. The pro-apoptotic activity of these drugs was attenuated in the presence of platelets.

What mechanisms can potentially be responsible for the effect of platelets? Firstly, platelets may limit the access of chemotherapeutic agent to cancer cells. Secondly, platelets may provide an anti-apoptotic mechanism to counteract pro-apoptotic effects of anticancer drugs. The first possibility appears less likely since Strieth and colleagues did not find significant interactions between platelets and paclitaxel in vitro (Strieth, Nussbaum et al. 2008).

Interestingly, I have found that both platelet releasate and intact platelets have the capacity to protect cancer cells from chemotherapeutic agent-induced apoptosis; however the degree of protection offered by whole platelets were larger than that of releasate. Platelets during activation release factors known to

modulate apoptosis. For example, growth factors such as VEGF and PDGF are known to regulate apoptosis in different cancer cell lines (Katoh, Tauchi et al. 1995), Furthermore, platelets of cancer patients contain high amounts of VEGF (Salven, Orpana et al. 1999; Folkman 2003).

There is also evidence that the factors associated with platelet membranes can modulate apoptosis. Indeed, human platelets contain abundant quantities of Fas-L upon activation and they express it on their surface as well as they release it into medium. This surface-expressed Fas-L is biologically active and can induce apoptosis in Fas positive human tumour cells (Ahmad, Menezes et al. 2001). Furthermore, the interactions between platelet surface membrane receptors such as glycoprotein IIb/IIIa, Ib, Ialla and P-selectin play a role in mediating platelet-cancer cell interactions (Janowska-Wieczorek, Majka et al. 2001; Baj-Krzyworzeka, Majka et al. 2002; Jurasz, Alonso-Escolano et al. 2004; Janowska-Wieczorek, Wysoczynski et al. 2005; Janowska-Wieczorek, Marquez-Curtis et al. 2006; Dean 2009). Moreover, Janowska-Wieczorek et al. demonstrated that platelet-derived microparticles transfer various surface receptors and adhesion molecules to target cells and modulate their biological response such as proliferation, survival and adhesion of human normal and malignant hematopoietic cells. Interestingly, activated platelet microparticles appear to have a significant impact on survival of patient suffering for prostate cancer, treated with docetaxel based chemotherapy. Indeed, The high level of microparticles is a predictive factor of poor prognosis of patient's survival (Helley, Banu et al. 2009).

As cancer apoptosis is regulated by a very large pool of genes I selected the TILDA method (TaqMan® Gene Expression Assays) to evaluate the expression of genes involved in apoptosis. In my analysis I focused on upregulation or

downregulation of genes most relevant to apoptosis. Platelet activation and interaction with Caco-2 cells, in the presence of paclitaxel, led to upregulation of gene expression, which are responsible for anti-apoptotic function such as IKBKG (NEMO), BRIC5 (survivin), NFκB2, REL and pro-apoptotic function such as APAF1, CASP6, BNIP3L. A similar experiment performed with 59M cells, in the presence of paclitaxel, showed downregulation of pro-apoptotic gene expression such as CASP2, DAPK1, LRDD, NALP1 and PYCARD and upregulation of pro-apoptotic genes BIK, CASP6, NFκBIA, NFκBIE and anti-apoptotic factors such as BCL3, RIPK2, NFκB1. All these data indicate that **platelets have the capacity to modulate the balance between pro-apoptotic and anti-apoptotic genes** presumably tipping the net balance towards apoptosis inhibition. For example, upregulation of NFκB2 and NFκB1 indicates the involvement of the anti-apoptotic pathway of NFκB in this effect of platelets. In keeping with this notion, Jenssens et al. demonstrated that LRDD (PIDD, p53-induced protein with a death domain) is important for activating an anti-apoptotic pathway involving the transcription factor NF-κB in response to genotoxic stress (Janssens, Tinel et al. 2005). PIDD form the complex that contains RIP1 (receptor-interacting protein 1) and NEMO, which are critical players in the signalling pathways that led to activation of the transcription factor NF-κB and cell survival by several DNA-damaging agents (Huang, Wuerzberger-Davis et al. 2003; Hur, Lewis et al. 2003). PIDD also form complex with RAIDD and procaspase-2, which trigger activation of caspase-2 and finally cell death. PIDD is a switch hitter between life or death of cells depending on cellular conditions and the DNA damage (Campbell, Rocha et al. 2004; Wu, Mabb et al. 2005).

Proteomics

As I have mentioned before platelets have the capacity to release factors that modulate platelet cancer interactions including growth factors, matrix metalloproteinase, ADP or nitric oxide (Radomski, Jenkins et al. 1991; Radomski 1993; Jurasz, Alonso-Escolano et al. 2004). We have now used proteomics to study in a comprehensive way the secretome of proteins released during the interactions between platelets and Caco-2 cells in the presence of paclitaxel. As proteomics is a very powerful method that generate the "sea" of data, which often require years of careful analysis I have focused in this thesis on proteins, that in my opinion, are most relevant to apoptosis and carcinogenesis. This initial analysis has already yielded some interesting observations identifying the set of 66 proteins. Some of these proteins are often considered as platelet-specific such as GPIIb/IIIa or GPIb/V. The presence of such proteins may indicate contamination of cancer cell samples with platelet products present in the culture medium (e.g. foetal bovine serum). However, the expression of platelet-specific proteins has been shown in some human cancer cells including breast cancer (Alonso-Escolano 2004).

The mass spectrometry analysis identified two proteins that may act as apoptosis inhibitors i.e. **serpin peptidase inhibitor** and **heat shock 70 kDa protein (Hsp70)**. Serpins are one of the largest and most diverse groups of protease inhibitors (Rawlings, Tolle et al. 2004) with various biological functions. Interestingly, Rousalova and colleges suggested that upregulated expression of human proteinase inhibitor-9 (PI-9)/serpinB9 in non-small cell lung carcinomas cells may protect them from apoptosis induced by granzyme B (Rousalova

2010). Serpin peptidase inhibitor clade A member 1 also can be used as a marker for malignancy in insulinomas (de Sá, Corrêa-Giannella et al. 2007).

I have also found that the **heat shock 70 kDa protein** (Hsp70) is released during platelet-Caco-2 cell interactions. This protein is involved in cytoprotection and molecular chaperoning. The Hsp70 may also act as potential oncogenic apoptosis inhibitor (Garrido 2003). Tran et al. suggest that cytoprotector Hsp70 could improve the environment for rapid cell growth even under stressed conditions (Tran, Brazeau et al. 2006).

The proteomics identified also four groups of proteins that can affect cancer cell survival.

The first group of identified proteins were factors involved in cell adhesion including **integrin, beta 3** (platelet glycoprotein IIIa, antigen CD61); **cadherin 17** and **thrombospondin 1**. Integrin beta 3, a part of alpha 2 beta 3 platelet receptor expressed also by some cancer cells plays a crucial role in TCIPA (Jurasz, Alonso-Escolano et al. 2004). Cadherins are glycoproteins involved in calcium-mediated cell-cell adhesion and epigenetic derangement of E-cadherin has been implicated in the pathogenesis of breast cancer (Cowin, Rowlands et al. 2005). Ichii et al. show that thrombospondin-1 accumulation at the smooth muscle cell (SMC) surface mediates the cell proliferation induced by direct interaction with platelets (Ichii, Koyama et al. 2002). Furthermore, thrombospondin-1 acts as an inhibitor of the soluble gunylyl cyclase in platelets increasing platelet activation (Miller 2010).

In the second group of identified proteins, there was a growth factor such as **latent transforming growth factor beta (TGF beta) binding protein 1** (Wierzbicki 2006). Transforming growth factor can act as growth stimulator or inhibitor and exert various physiological and pathological functions including

cancer (Wong 2001). Interestingly, TGF beta is synthesised as latent protein and requires activation and beta 3 integrins and thrombospondin-1 can activate latent TGF beta (Murphy-Ullrich 2000; Wipff and Hinz 2008).

The third group of identified proteins are proinflammatory molecules including **chemokine (C-C motif) ligand-5 (CCL5 or RANTAS)**, **platelet factor-4 (PF-4)**, **platelet basic protein precursor (CXCL7)**. Platelet factor 4 is one of platelet alpha granule proteins. Cervi and colleagues reported that PF-4 is a biomarker in early stage of tumour growth. Moreover, PF-4 is upregulated in early growth of human liposarcoma, mammary adenocarcinoma, and osteosarcoma (Cervi, Yip et al. 2008). RANTES is also produced by tumor cells and promotes their survival, proliferation and invasion in e.g. prostate, breast and ovarian cancer. Moreover, the expression level of RANTES is positively correlated with the severity of the diseases (Niwa 2001).

The fourth group of identified proteins were **peroxiredoxins 3, 4** and **5**. These proteins effectively scavenge peroxide and may also play a role in mitochondrial redox signalling (Cox 2009). These proteins act to enhance cell survival under conditions of oxidizing stress. De Simoni et al. investigated the expression and function of peroxiredoxin in human neuroblastoma cells showing that decreased expression of periredoxin is associated with increased oxidative damage (De Simoni, Goemaere et al. 2008).

Cell cycle and Cyclins

Cell cycle machinery controls cell division and this process is aberrant in cancer. Fundamentally, all cancers permit the existence of too many cells. However, this cell number excess is linked in a vicious cycle with a reduction in sensitivity to

signals that normally tell a cell to adhere, differentiate, or die. This combination of altered properties increases the difficulty of deciphering the changes, which are primarily responsible for causing cancer and chemoresistance (Collins, Jacks et al. 1997).

Having obtained the data showing that platelets protect Caco-2 and 59M from apoptosis I have designed experiments to investigate if platelets affect cancer cell cycle under conditions of chemotherapeutic challenge. The present study describes a unique in vitro co-incubation experiment, which aims at examining interactions between platelets and colonic and ovarian cancer cells during their cycle phase progression. For this purpose I analysed cell distribution to particular phases G_0/G_1, S, G_2/M in untreated and treated cells by paclitaxel or 5-fluorouracil. Interestingly, in the presence of platelets less 59M and Caco-2 cells were in phase G_0/G_1 suggesting that **platelets help cancer cells bypassing irreversible cell cycle arrest - cellular senescence**. In contrast, in the presence of platelets, higher numbers of cancer cells were detected in phases S and G_2/M showing that **platelets have the capacity to stimulate DNA synthesis in cancer cells challenged with anticancer drugs**.

To study mechanisms responsible for these effects of platelets I have investigated the role of cyclins. The progression of a cell through the cell cycle is promoted by cyclin-dependent kinases (Cdks), which are positively regulated by cyclins and negatively regulated by cyclin-dependent kinase inhibitors. Therefore, I investigated the effects of platelets on the expression of cyclin A, B1, D1 and E as they regulate the cell cycle progression. There are two types of cyclin A, cyclin A1 that binds to Cdk2 and the cyclin A1/Cdk2 complex is responsible for S-phase progression (DNA synthesis). Cyclin A2 forms complex with CDC2 gene and this complex known as Cdk1 is important for G_2

phase. Interestingly, cyclin A binds both cdk2 and CDC2, giving two distinct cyclin A kinase activities, one appearing in S phase, the other in G_2/M in the human cell cycle (Pagano 1992). The synthesis of cyclin A is mainly controlled by E2F and other transcription factors. Both cyclin A1 and cyclin A2 enhance double-strand break (DSB) repair by homologous recombination, but only cyclin A1 significantly activates nonhomologous end joining, DNA repair pathway. Cyclin A1 is induced by γ-irradiation via a p53-mediated mechanism (Muller-Tidow, Ji et al. 2004). I found **a significant upregulation of cyclin A in the presence of platelets** in 59M untreated and 5-fluorouracil-treated cells, in all phases of cell cycle. In contrast, platelets did not modify regulation of cyclin A in paclitaxel - treated cells. This may be explained by direct action of paclitaxel on cyclin A (Perez-Stable 2006). Cyclin A is strongly related to cancer cell cycle as this protein is elevated in a variety of tumours (Yam 2002). Wegiel et al. have shown that overexpression of cyclin A1 increased migration and invasiveness of prostate cancer in vitro and promoted metastasis. The importance of cyclin A1 in tumour cell invasion and metastasis is suggested by its high level of nuclear expression in aggressive prostate cancer and its ability to mediate the activities of MMPs and VEGF in cultured cells (Wegiel 2008). Elevated levels of cyclin A1 expression have been observed in various types of solid tumours, including testicular, ovarian, and breast tumours (Rivera 2006). Another research group examined the effect of nitric oxide (NO) on the proliferation and cell cycle regulation of human aortic vascular smooth muscle cells (VSMCs). NO inhibits VSMC proliferation by inducing G_1 phase arrest due to specific changes of the expression and activity of cell cycle regulatory proteins. NO synthase (NOS) overexpression did not inhibit proliferation in response to platelet-derived growth factors (Tanner, Meier et al. 2000).

Cyclin B1 is one of the main components of the cell cycle machinery. Cyclin B1 binds to CDC2 forming an activated cyclin B1/Cdc2 complex, which regulates G_2/M transition of cell cycle thus promoting cell mitotic division (Jin, Hardy et al. 1998). Overexpression of cyclin B1 has been reported in various human tumours, such as breast cancer, cervical cancer, gastric cancer, colorectal cancer and non-small-cell lung cancer (Wang 1997; Soria, Jang et al. 2000; Zhao 2006). Similar to cyclin A data, I have found a **significant upregulation of cyclin B1 in the presence of platelets** in 59M cells treated with 5-fluorouracil, but not with paclitaxel. Cyclin B also plays an important role in cancer cell cycle. Aaltonen et al. analysed cyclin B1 in 1348 invasive breast cancers and studied correlations with other histopathological variables and survival. They reported the association of high cyclin B1 and shorter survival in breast cancer patients. The study showed also that cyclin B1 is an independent predictor of poor overall prognosis and metastasis-free survival in breast cancer. The generally lower risk ratios for mortality or metastases in patients given adjuvant chemotherapy suggest that high cyclin B1 score may indicate an enhanced sensitivity to chemotherapy (Aaltonen, Amini et al. 2009).

Cyclin D is an important regulator of G_1 to S phase progression during cell cycle. Together with its binding partners, cyclin-dependent kinase 4 and 6 (Cdk4 and Cdk6), cyclin D1 forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (Rb) (Weinberg 1995; Alao 2007). While there are three different D-type cyclins, only cyclin D1 is overexpressed frequently in cancer. Cyclin D1 plays a role in the development and progression of several cancers including breast, oesophagus, bladder and lung cancers (Gillett 1996; Yamamoto, Tamakawa et al. 2006; Alao 2007). Overexpression of cyclin D1 has also been linked to the development of

endocrine resistance in breast cancer cells (Kenny, Hui et al. 1999). In addition, cyclin D1 has cell cycle-independent functions through its ability to modulate the actions of transcription factors (Coqueret 2002). Cyclin D1 is also pro-survival factor for a number of different cell types (Schwartz and Shah 2005). Similar to cyclins A and B1 I have found **a significant upregulation of cyclin D1 in the presence of platelets** in 59M untreated cells (phase S and phase G_2/M) and in 5-fluorouracil-treated cells in phase S; but this effect was not detected in paclitaxel-treated cells. Igawa et al. transfected platelet-derived endothelial cell growth factor (PD-ECGF) into the head and neck squamous cell carcinoma cell line IMC-3 and investigated the property of transfectants in vitro. The results showed that the cancer cell line with high expression of PD-ECGF had a rapid cell cycle and consequently facilitated rapid cell growth, not only in vitro, but also in vivo. The expression of cyclin D1 and cyclin E were more enhanced in PD-ECGF transfectants than parental cells. Moreover, in PD-ECGF transfectants cells, S and G_2/M phases were rapidly increased compared with parental cells (Igawa 2003). Kohama et al. observed that platelets expressing TGF-β3 led to increased expression of cyclin D1 and tenascin-C, and increased mesenchymal cell proliferation (Kohama, Nonaka et al. 2002).

Finally, I have studied the effects of platelets on cyclin E. There are two types of cyclin E, E1 and E2, which are expressed during the G_1 and S phases (Dulić 1992; Ekholm, Zickert et al. 2001). Cyclin E plays a crucial role in the initiation of DNA replication as it regulates the S phase entry (Ohtsubo, Theodoras et al. 1995). Cyclin E binds cyclin-dependent kinase Cdk2 and forms the cyclin/Cdk2 complex, which initiates a cascade of events leading to the expression of S-phase. Expression of cyclin E is regulated on the level of transcription factors, mainly by E2F (Möröy and Geisen 2004).

In addition, high level of cyclin E expression is present in different human cancers, in particular breast cancer, leukemia, lymphoma and others (Lingle, Lutz et al. 1998; Hwang and Clurman 2005). However, overexpression of cyclin E decreases mobility and invasiveness of breast cancer cells (Berglund 2006). I have found **significant upregulation of % of cyclin E expression in the presence of platelets** in 59M untreated cells, but not in cells treated with paclitaxel. In 5-fluorouracil-treated cells, platelets exerted similar (albeit statistically non-significant) effect on cyclin E. Thus the significance of increased cyclin E expression remains to be investigated.

DNA Damage Repair

The genome is constantly exposed to agents, which damage DNA. In the absence of DNA repair, the genome would be unable to survive. If DNA damage is left unrepaired, this may lead to DNA instability and the consequential risk of cancer and other pathologies. Therefore, a range of molecular mechanisms and proteins are involved in the response to DNA damage. These proteins coordinate the repair of DNA lesions and the stalling of the cell cycle to allow repair to occur (Martin, Lord et al. 2008). DNA damage induces several cellular responses including DNA repair, checkpoint activity and the triggering of apoptotic pathways (Niida and Nakanishi 2006). The induction of double-strand DNA breaks (DSBs) by genotoxic agents provides a signal for histone H2A.X phosphorylation on Ser139 (γH2A.X) to rapid activation on chromatin surrounding the DNA damage (Huang, Halicka et al. 2005; Tanaka, Huang et al. 2007; van Attikum and Gasser 2009). Some of the key proteins recognize the DNA breaks, for example the Mre11 complex (Mre11/Rad50/Nbs1) (MRN) and

ataxia-telangiectasia (A-T) mutated (ATM). The MRN complex activates a variety of other proteins involved in cell cycle control and DNA repair. The Mre11/Rad50/Nbs1 complex is involved in both homologous and non homologous repair of double-strand breaks (Lavin 2004; Lavin 2007). Telomeres are protected and maintained by specific proteins and Mre11/Rad50/Nbs1 complex is also involved in this function (Zhang, Zhou et al. 2006; Ueno. 2010). The DNA damage checkpoints Chk1 and Chk2 Ser/Thr kinases and Cdc25 phosphatises activate p53 and inactivate cyclin-dependent kinases to inhibit cell cycle progression from G_1 to S (the G_1/S checkpoint), DNA replication (the intra-S checkpoint), or G_2 to mitosis (the G_2/M checkpoint) (Sancar, Lindsey-Boltz et al. 2004). The activation of these checkpoints allows repair of DNA damage, before it is replicated and passed on to daughter cells and therefore preserves the genomic integrity (Bolderson, Richard et al. 2009). BRCA1 is a nuclear protein with a cell cycle-regulated expression pattern, responsible for DNA repair process and it is very often mutated in breast and ovarian cancer. BRCA1 is hyperphosphorylated in response to DNA damage and co-localizes with Rad51, a protein involved in homologous recombination, and Mre11/Rad50/Nbs1 repair the damaged of DNA (Gatei, Scott et al. 2000; Kim 2008). BRCA1 and BRCA2 appear to be functionally related to DNA repair mechanisms (Kinzler 1997). BRCA1 plays a critical role in the DNA damage recognition and in cell cycle checkpoints control that allow cell cycle progression only after DNA repair, avoiding genetic damage transmission in subsequent cell generations (Kennedy, Quinn et al. 2004). There are different repair pathways of DNA damage such as homologous recombination (HR), non-homologous end joining (NHEJ), base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) and their activation depending on type of DNA damage.

Indeed, the interaction between the DNA repair pathways is very complex and not yet fully understood (Martin, Lord et al. 2008). Inhibitors of DNA repair proteins for example ATM, BRCA1, Chk1, PARP, DNA-PK have been used in cancer therapy, mostly to potentiate the effects of cytotoxic agents. However, tumour cells frequently exhibit deficiencies in the signalling or repair of DNA damage. These deficiencies probably contribute to pathogenesis of the disease, but they also present an opportunity to target the tumour.

One of the main pharmacological properties of anticancer drugs is to induce DNA damage in the target cells. Furthermore, pharmacologically inhibiting DNA repair components in cancer cells may also be used to enhance chemosensitivity and radiosensitivity (Dasika 1999; Ding, Miao et al. 2006; Martin, Lord et al. 2008). Indeed, inhibitors of DNA repair proteins may be crucial to achieve better effects of chemotherapy (Shapiro and Harper 1999; Ashwell and Zabludoff 2008; Martin, Lord et al. 2008).

To determine whether platelets exert any effect on DNA repair in 59M and Caco-2 cells exposed to 5-fluorouracil and paclitaxel. I have used Western blotting to assay DNA repair proteins such as BRCA1, checkpoint 1, Mre11 and p95/Nbs1. I have found that active (phosphorylated) BRCA1 was increased in the presence of platelets in both cell lines challenged with 5-fluorouracil and paclitaxel. Similar results were obtained with phospho-checkpoint 1, phospho-Mre11and phospho-p95/Nbs1 for 59M cells. The results obtained from Caco-2 cells were not statistically significant. All these results taken together show those platelets have the ability to increase amount of active part of different DNA repair proteins when DNA damage occur.

MAP Kinases

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases which mediate extracellular signals and control the crucial cellular processes such as proliferation, differentiation, survival, death, and migration (Dhillon, Hagan et al. 2007). MAPK signalling pathways have been implicated in the pathogenesis of a variety of human disorders including cancer and neurodegenerative diseases such as Alzheimer and Parkinson diseases. There are three predominantly MAPK pathways in mammals: extracellular signal-regulated kinase (ERK), p38 and c-Jun NH_2-terminal kinase (JNK). Upon phosphorylation they are activated by a mitogen-activated protein kinase kinase kinase (MAPKKK) which change to MAPK kinase (MAPKK) and finally to the MAPK. Each of the pathways has several isoforms: ERK1 to ERK8; p38-α, -β, -γ, δ and JNK1 to JNK3 (Schaeffer and Weber 1999). The ERK1 and ERK2 pathways are stimulated by growth factors and mitogens which get activated through phosphorylation of Raf (Raf-1, B-Raf and A-Raf). ERK pathway is deregulated in one-third of all human cancers (Dhillon, Hagan et al. 2007). In many types of cancer there occurred mutations of components of ERK signalling pathway such as Ras and B-Raf (Boutros 2008). K-Ras mutation is very often present in human lung and colon cancer (Schubbert, Shannon et al. 2007; Haigis, Kendall et al. 2008). Mutations in the B-Raf and Raf1 genes occurred in majority of malignant melanomas (Wan, Garnett et al. 2004; Rushworth, Hindley et al. 2006). Indeed ERK1,2 pathway plays also role in diverse stages of cancer development such as upregulated expression of matrix metalloproteinase, which consequently improve tumour invasion (Chakraborti 2003). Moreover, this pathway increases the expression of anti-apoptotic factors MCL-1 as promoting the survival of cancer cells

(Balmanno and Cook 2008). Therefore ERK1, 2 pathway is a prominent therapeutic target for the development of chemotherapeutic drugs. The mutation was detected in the epidermal growth factor receptor (EGFR), which activates the ERK1, 2 pathway. The abnormal upregulated activity has been observed in colonic and in 80% of non-small cell lung cancer (Nagahara, Mimori et al. 2005; Dy and Adjei 2009). The inhibitors of the tyrosine kinase activity of EGFR are targets for the development of new therapeutic drugs for lung cancer (Jian-Quan, Wen-zhao et al. 2008). The JNK and p38 signalling pathways are activated by pro-inflammatory cytokines and cellular stresses such as genotoxic, osmotic, hypoxic, lipopolysacharide (LPS) or oxidative stress (Weston and Davis 2002; Kim and Choi 2010). In response to DNA damage, JNK pathway activated p53 (Wu 2004). Interestingly, JNK signalling - activation of nuclear factor kappa B (NF-κB) signalling can lead to the suppression of apoptosis (Bubici 2004). JNK and NF-κB signalling very often play opposite roles (Kennedy 2003). Inhibition of this transcription factor may increase cancer cell apoptosis. The p38 pathway is involved in activation of p53 and induced apoptosis. Moreover p53 is a negative regulator of cell cycle progression (Bulavin and Fornace 2004; Bradham 2006). p38 activity was reduced in hepatocellular carcinomas in comparison to the normal tissue (Iyoda, Sasaki et al. 2003). A lot of chemotherapeutic agents require p38 activity pathway to induce apoptosis such as cisplatin, doxorubicin and taxol (Losa, Cobo et al. 2003; Lee, Kim et al. 2006).

To determine a potential involvement of MAPKs in the effects of platelets on 59M and Caco-2 cells challenged with 5-fluorouracil and paclitaxel I assayed phosphorylated p38, p42/44, JNK 54 and JNK 46 using Western blot. The results indicate that platelets have the capacity to upregulate these proteins both

in 59M and Caco-2 cells. These effects could contribute to increased survival of cancer cells in the presence of platelets. Interestingly, Baj-Krzyworzeka et al. showed, that peripheral blood platelet-derived microparticles (PMPs) circulated in blood and might interact with various cells affecting their diverse biological functions. They found out that, PMPs express platelet-endothelium attachment receptors such as CD41, CD61, CD62 and different G-protein-coupled seven transmembrane-span receptors for example: CXCR4 and PAR-1; cytokine receptors including TNF-RI, TNF-RII, CD95; and ligands such as CD40L and PF-4. Moreover, they found out that these receptors could be transferred by PMPs to the membranes of normal and malignant cells. It was observed that PMPs chemo attract these cells, increase their adhesion, proliferation, survival and activate in these cells various intracellular signalling cascades including MAPK p42/44, PI-3K-AKT, and JNK proteins (Baj-Krzyworzeka 2002).

Telomerase

Human telomerase is a ribonucleoprotein enzyme complex that enables cells to maintain telomere length, which plays a fundamental role in genome maintenance and stability. Telomerase has been regarded as one of the most promising therapeutic target for novel anticancer interventions (Tian 2010). Many factors contribute to this issue, such as its high expression level in cancer cells. Moreover, cancer cells have shorter telomeres than normal cells and a more rapid cellular proliferation rate. It means that telomerase inhibition strategies would have a greater and more significant impact on the survival of cancer cells while having minimal side effects on normal somatic cells. Some research studies have indicated that telomerase inhibition leads to apoptosis of

cancer cells or inhibition of cell proliferation (Hodes. 2001; Folini M 2002; Ahmed and Tollefsbol 2003; Chen 2009).

To determine whether platelets have the effect on telomerase activity in Caco-2 cells, the cells were incubated with 5-fluorouracil in the presence or absence of platelets. Unfortunately, under experimental conditions used the drug did not significantly affect telomerase activity; therefore, I am not in a position to comment on the effects of platelets in this system. Interestingly, Schröder et al. studied leukocyte telomere length and telomerase activity before and after treatment in breast cancer patients. Haemoglobin, MCV, leukocyte and platelet numbers were assessed prior to (t_0), 5 months after (t_1) and 9 months after chemotherapy (t_2). They reported that there is not any correlation between the haematological parameters such as haemoglobin, MCV, leukocyte and platelet counts at t_1 and t_2 and TRF length or Δ TRF length. The measurement of telomerase activity at t_0 and t_1 was also performed. However, no strong upregulation of telomerase activity was observed, also not in patients with increased TRF lengths after treatment (Schroder, Wisman et al. 2001).

Pharmacological and Clinical Significance

My results are relevant to the understanding of the pharmacological and clinical profile of paclitaxel and 5-fluorouracil. Paclitaxel is a unique anti-microtubule agent that acts by inhibiting microtubule depolymerisation and thereby disrupting the normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation. Drug blocks cells in the G_2 and M phase of the cell cycle and stabilizes cytoplasmic microtubule (Schiff 1980; Sui, Dziadyk et al. 2004). 5-Fluorouracil is a pyrimidine analogue, inducing cell cycle arrest and

apoptosis by inhibiting the cell's ability to synthesize DNA. It is an S phase specific drug and only active during certain cell cycles by cytotoxic metabolites, which are later incorporated into DNA and RNA (Schuetz, Wallace et al. 1984). As I have shown that the anticancer activity of these drugs is reduced in the presence of platelets this phenomenon may explain, in part, the resistance of ovarian and colonic cancers to the treatment with these agents. The data points out also to possible mechanism involved in this process.

Firstly, platelets upregulate anti-apoptotic genes such as **nemo, survivin, NFκB2** and downregulate pro-apoptotic ones such as **APAF1, CASP6, BNIP3L**. Platelets also have the effect on regulation of **cell cycle phases**. The Caco-2 and 59M cells have predominated distribution in S and G_2/M phases during the cell cycle in the presence of platelets as platelets being in better condition for DNA synthesis and cell division of the mother cell into two daughter cells. Furthermore, cells in the presence of platelets bypass irreversible cell cycle arrest. Moreover, the anucleated elements upregulate expression of cell cyclins such as **A, B1, D1 and E**. Indeed, DNA repair proteins (**BRCA1, Chk1, Mre11, p95/Nbs1**) also increase their amount of phosphorylated part in the presence of platelets. **Figure 68** shows a summary of cancer pathways that are targeted by platelets.

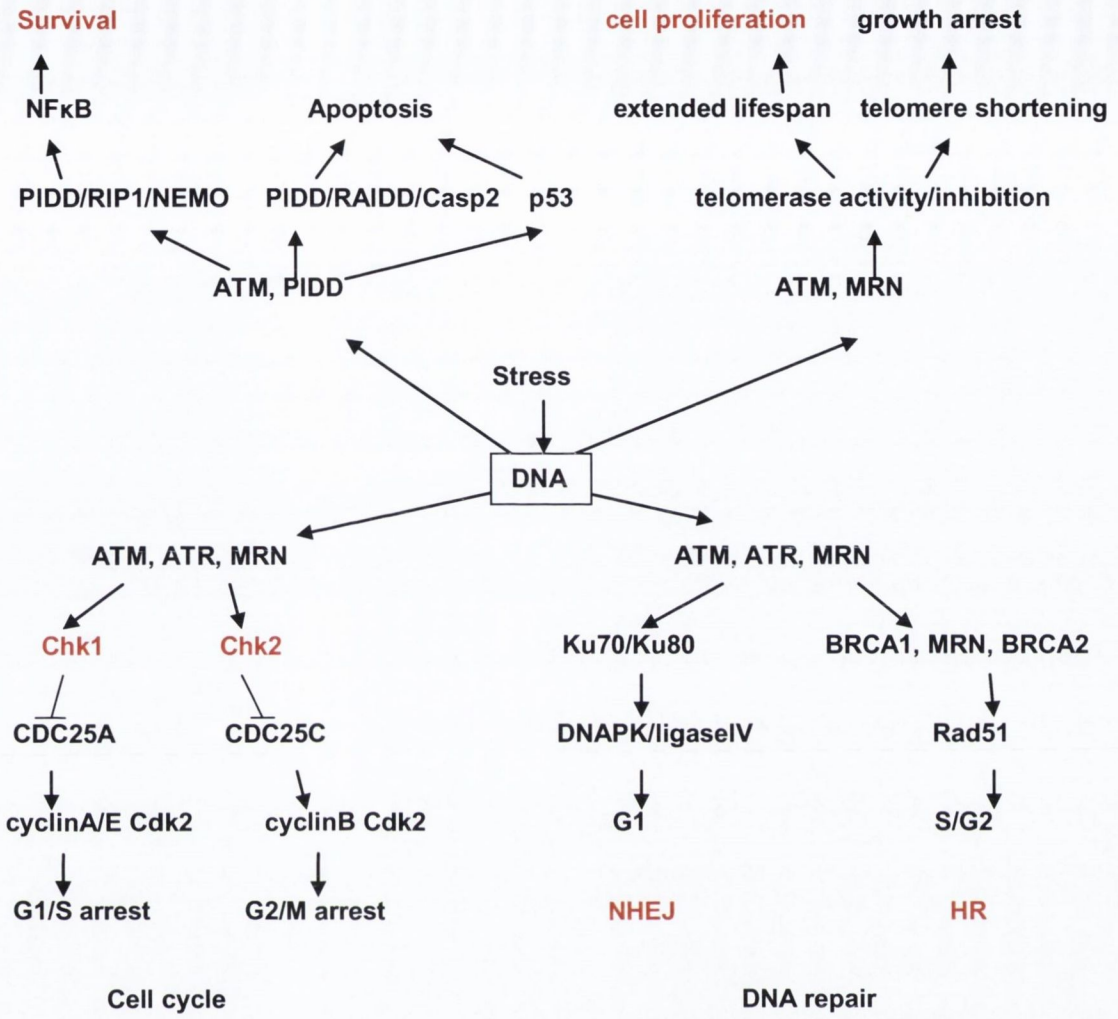


Fig. 68. Schematic pathways after DNA damage exogenous and/or endogenous. The red colour shows the molecular events modulated by platelets as found in my study. Adapted from (Gorbunova V 2003; Habraken and Piette 2006; Bolderson, Richard et al. 2009).

There is substantial pharmacological development targeting carcinogenetic mechanisms that has been shown to be controlled by platelets in my study. Flavopiridol is a novel inhibitor of cyclin-dependent kinases, which can induce cell cycle arrest in a number of cell lines (Carlson, Dubay et al. 1996). The agent has inhibited anti-apoptotic molecules including Bcl-2, XIAP, p21, mcl-1, cyclin D1, and phospho-survivin (Carlson, Lahusen et al. 1999; Wall, O'Connor et al. 2003; Castedo, Perfettini et al. 2004; Schwartz and Shah 2005). All three new clinical candidates XL-844, AZD7762 and PF-477736 (AstraZeneca) are potent

inhibitors of both Chk1 and Chk2 and they represent different chemical classes (Castedo, Perfettini et al. 2004; Ashwell, Janetka et al. 2008). In addition, MAPK pathways ERK1, 2; p38 and JNK are upregulated by platelets. In number of pharmaceutical companies, inhibitors of p38 MAPK pathway are in clinical trials, in vitro or in vivo stage (Yong, Koh et al. 2009). SB203580 inhibits colon cancer growth and induces apoptosis (Lim 2006). The same agent is investigated in breast cancer, where it triggers inhibition of TGF-β-induced invasion and migration (Kim 2004). Another inhibitor SB202190 reduces AP-1 activity and increases the sensitivity of chemotherapy in gastric cancer (Guo, Ma et al. 2008). Several drugs are in clinical trials, which target the inhibition of the telomerase enzyme and proteins involved in the telomere/telomerase complex. They include the heat-shock protein 90 inhibitor 17-allylaminogeldanamycin, which shows downregulating telomerase activity (Villa, Folini et al. 2003; Fletcher 2005), and PARP inhibitory compounds (Shay and Wright 2005; Phatak 2007).

These results also underlie the importance of designing novel approaches to inhibit "the fatal interplay between platelets and cancer" (Jurasz, Alonso-Escolano et al. 2004; Erpenbeck and Schon 2010). Although a great number of platelet-dependent mediator/mechanisms have been pharmacologically targeted including platelet receptors (GPIb, GPIIb/IIIa, PAR receptors and P-selectin) and soluble factors (thrombin, ADP and thromboxane) cancer cells display remarkable arsenal of pathways that can bypass the pharmacological actions of platelet inhibitors (Jurasz, Alonso-Escolano et al. 2004). Thus, despite of years of intensive research "the magic bullet" to target platelet-cancer interactions remains to be found……

FUTURE DIRECTIONS

I feel that future directions of my research should address potential limitations of my PhD studies:

1. It would be important to extend the core observation i.e. protective effects of platelets on anticancer drug-induced cell damage to different anticancer drugs and human cell lines. In my study I have selected to human cell lines 59M (ovarian cancer) and Caco-2 (colonic cancer) that have been challenged with paclitaxel and 5-fluorouracil drugs specifically used in the treatment of both cancers. The two drugs, although chemically unrelated and with different mechanism of action target cell cycle. It would be interesting to extend the observations using different groups of anticancer drugs. Similarly, cell lines originating from different cancers could also be studied.

2. All my results have been obtained using in vitro experiments. This approach is very valuable as it enabled me to provide a proof-of-principle involving the multitude of mechanisms involved. It would be of importance to use an in vivo animal model of cancer to confront and verify the results obtained in vitro investigations.

3. Proteomics has generated the wealth of data. I would like to proceed further with data mining as thus approach is often used to identify targets for possible pharmacological interventions.

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