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der Ruprecht-Karls- Universität Heidelberg

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"My Lord! Increase me in Knowledge"

-Quran; 20:114

Dedicated to the soul of my father and to my mother, brother & sister for the endless love and support and to my sons

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List of abbreviations

ABC	Adenosine triphosphate- binding cassette
ABTS	2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid)
ADCC	Antibody-dependent cell mediated cytotoxicity
ALL	Acute lymphoblastic leukemia
ALM	Alemtuzumab
AML	Acute myeloid leukemia
AP	Alternative pathway
ATP	Adenosine triphosphate
B-CLL	B-chronic lymphocytic leukemia
BTZ	Bortezomib
BSA	Bovine Serum Albumin
C1-Inh.	C1-inhibitor
C4bp	C4-binding protein
CDC	Complement dependent cytotoxicity
CDCC	Complement-dependent cellular cytotoxicity
CHOP	(Cyclophosphamide, Hydroxydaunomycin, Oncovin, Prednisone)
CML	Chronic myeloid leukemia
CO_2	Carbon dioxide
CR1	Complement receptor 1
CR2	Complement receptor 2
CR3	Complement receptor 3
DAF	Decay-accelerating factor
ddH_2O	Double distilled water
DFS	Disease-free survival
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Di-Methyl Sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMMPRIN	Extracellular matrix metalloproteinase inducers
Ep-CAM	Epithelial cell adhesion molecule
ER	Estrogen receptor

ERK	Extracellularly regulated protein kinase
FACS	Fluorescence Activated Cell Sorting
FAP	Fibroblast activation protein
FBS	Fetal Bovine Serum
FcγRs	Fc gamma receptors
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
fH	Factor H
fI	Factor I
Fluda	Fludarabine
GST	Glutathione S-transferase
gt	Goat
	Hour
H_2O_2	Hydrogenperoxide
HCL	Hydrochloric acid
HNSCC	Head and neck squamous cell carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HMs	Hematologic malignancies
HRP	Horseradish peroxide
i.a. NHS	Inactive Normal Human Serum
ICD-O-3	International Classification of Diseases for Oncology, Third Edition
IgG	Immunoglobulin G
mAbs	monoclonal antibodies
MAC	Membrane attack complex
MASPs	Mannose-binding lectin -associated serine proteases
MBL	Mannose-binding lectins
MCL	Mantle cell lymphoma
mCRPs	Membrane-bound complement regulatory proteins
MDR	Multidrug resistance
mg	Milligram
Min	Minute(s)
µl	Microlitter
µM	Micromolar
ml	Milliliter
mM	Millimolar
ms	Mouse

NHL	Non-Hodgkin's lymphoma
NHS	Normal human serum
NK-cells	Natural killer cells
nM	Nanomolar
NSCLC	Non-small-cell lung carcinoma
OFA	Ofatumumab
OS	Overall survival
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
P-gp	P-glycoprotein
PI	Propidium Iodine
PKC	Protein kinase C
PLL	Prolymphocytic leukemia
PMNs	Polymorphonuclear leukocytes
PR	Progesterone receptor
RANKL	Receptor activator of nuclear factor kappa-B ligand
rb	Rabbit
RNAi	Ribonucleic acid- interference
rpm	Rotation per minute
RTX	Rituximab
SCR	Short consensus repeat
STS	Soft tissue sarcomas
Tax	Taxol
TNBC	Triple negative breast cancer
TME	Tumor microenvironment
TILs	Tumor infiltrating lymphocytes
VBS	Veronal-buffered saline
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

Chapter 1– Introduction

1.1 Cancer definition

Cancer is a complicated and multifactorial disease, in which normal cells undergo a series of molecular changes combined with accumulation of somatic mutations in progenitor cells resulting in a conversion into abnormal cells with uncontrolled growth and dynamic genetic alterations (MacConaill and Garraway, 2010). Tumor invasion of nearby and distant tissues impairs the normal biological processes of healthy cells in the microenvironment (Goldenberg, 1999).

1.2 Classification and epidemiology

The international classification of diseases for oncology (ICD-O) has been recognized as the gold standard for classification of neoplasms. The third edition of ICD-O (ICD-O-3) has been available since 2000 and an updated version with a number of new or modified codes and terms (ICD-O-3 first revision, or ICD-O-3.1) was published in 2013 (the world health organization; WHO, 2013).

Tumors have been classified according to either histological type or the primary site (first location of cancer in the body). Medical professionals gave cancer names based on their histological type. However, the general population is more familiar with cancer names based on their first location in the body. The most common body sites in which cancer develops include: skin, lungs, female breasts, prostate, colon and rectum, cervix and uterus (SEER Training Modules, Cancer classification, US. National Institute of Health, National Cancer Institute. *21th July, 2018. https://training.seer.cancer.gov/*). Defined cancers based on the primary site are not as accurate as those defined based on the histological type. In lung cancer, for example, the name does not specify the involved tissue of the lung, but simply indicates where the cancer is actually located. Therefore, based on how cells look like under a microscope, there are two major types of lung cancer: non-small cell lung cancer and small cell lung cancer. Non-small cell lung cancer can be further divided into various subtypes which

include squamous cell carcinoma, adenocarcinoma and large cell carcinoma (Inamura, 2017).

Histologically-defined cancer types are grouped into six major categories: carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed types. The worldwide burden of non-communicable diseases is growing in recent years and the main reasons are: prolonged exposure to risk factors, increased life time, and changes in lifestyle (Razi *et al.,* 2015; Zahedi *et al.,* 2015). Concerning cancer, in 2012, documented tumor cases accounted for ~14.9 million, of which 4.3 million patients died. By 2030, an increase of this number by 44% is expected (Siegel *et al.,* 2017) and this number may increase to 22 million in two decades (Ferlay *et al.,* 2015a). In 2018, 1.735.350 new cancer cases and 609.640 cancer deaths were calculated in the United States of America USA; (Siegel *et al.,* 2018). Lung, breast, prostate, cervix, colon, stomach and liver cancers are the most common diagnosed types throughout the world (Parkin e*t al.,* 2002). Here, we are going to shed more lights on hematologic and breast cancers.

1.2.1. Hematologic malignancies

Hematologic malignancies (HMs; blood cancer) originate either in bone marrow or immune cells and comprise a distinct group of malignancies with variable etiology, incidence, prognosis and survival rates (Rodriguez-Abreu *et al.,* 2007; Sant *et al.,* 2010).

The WHO has approved a consensus classification for HMs which relies on cell lineage (myeloid and lymphoid) and cell maturity and for further subdivision, cell morphology, immunophenotype, genetic and clinical criteria were applied (Vardiman, 2010). In adition, HMs are grouped into different categories including Hodgkin (HL) versus non-Hodgkin lymphoma (NHL), acute versus chronic, and lymphatic versus myeloid leukemia (Ferlay et al., 2007; Curado et al., 2007). In 2005, the estimated HMs cases in Europe were ~230.000. Leukemia, HL, NHL and myeloma accounted for ~8% of HMs-diagnosed patients with a ~7% death rate (Rodriguez-Abreu et al., 2007). In 2017, from 1.688.780 confirmed cancer cases in the USA 10.2% were diagnosed with leukemia, lymphoma and myeloma and the estimated number of fatalities due to these tumors was 58.300 (Cancer Facts and Figures, 2017).

1.2.2. Breast cancer

Breast cancer starts in breast cells as a group of cells that can then invade surrounding tissues or spread (metastasis) to other body parts. It is the second most common cancer all over the world and in absolute numbers the most frequent cancer among women with an estimated 1.67 million of the new cancer cases diagnosed in 2012 (25% of all cancers; Clegg *et al.,* 2009; Ferlay *et al.,* 2015). The incidence of breast cancer varies nearly fourfold across the world regions, with incidence rates ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 96 in Western Europe (Ferlay *et al.,* 2015; WHO, 2015). In most countries, breast cancer representes 25% of all annualy diagnosed cancer types and is amongest main causes of women death around the world (Fitzmaurice *et al.,* 2015). Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) and while it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung (Ferlay *et al.,* 2015). Based on the presence of defined biomarkers like estrogen receptor (ER), progesterone receptor (PR), Ki-67 (a protein marker with prognostic and predictive potential for adjuvant chemotherapy), and human epidermal growth factor receptor 2 (HER2), breast cancer has been classified into several sub-types (Inic *et al.,* 2014). The most common receptors of breast cancer cells belong to the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (Nuciforo *et al.,* 2015). Over-expression of EGFR and HER2 was observed in approximately 40% and 25% of breast cancer cases, respectively, and is responsible for both aggressive tumor behaviour and poor prognosis (Nuciforo *et al.,* 2015). Moreover, triple negative breast cancer (TNBC) is defined by the absence of ER, PR, and HER2 receptors and no treatment is yet available for TNBC, which confers an unfavourable prognosis for this particular subtype (Gluz *et al.,* 2009).

1.3. The complement system

Complement was discovered more than 100 years ago as a heat-labile component of normal plasma that intensifies opsonisation and killing of invading microorganisms by antibodies (Janeway et al., 2001 (5th edition)). Complement proteins are mainly synthesized in the liver. However, they can also be

produced by various cell types such as macrophages, fibroblasts, and endothelial cells (Macor and Tedesco, 2007a). It is composed of more than 30 glycoproteins that are either present in plasma or associated with the cell membrane as regulators or receptors (Ricklin *et al.,* 2010; Noris and Remuzzi, 2013).

Although complement was first defined only as an effector arm of the antibody response, it also can be activated at early stages of infection in absence of antibodies. The immune effector functions of complement include cell lysis, opsonisation, chemotaxis and immune cell activation (Herceg and Hainaut, 2007). Given the numerous genetic and epigenetic changes associated with carcinogenesis, neoplastic transformation is also accompanied by an increased capacity of malignant cells to activate the complement system (Herceg and Hainaut, 2007).

1.3.1. Activation pathways

There are three major complement activation pathways (Figure 1): the classical pathway (CP) via antigen-antibody complexes, the alternative pathway (AP) through any permissive surface and lectin pathway (LP) by the interaction of pattern-recognition mannose-binding lectins (MBL) or ficolins with carbohydrate ligands on the surface of the pathogens (Frank, 1987; Jack *et al.,* 2001; Turner, 2003; Holers, 2014).

The convergence point of complement activation pathways is the formation of the C3 convertase which cleaves the complement C3 molecules into C3a (one of two major anaphylatoxin molecules) and C3b, which is a potent opsonin (Ricklin *et al.,* 2010). C3b and its degradation products iC3b and C3d bind to intact target cells or cell debris and act as ligands for complement receptors CR1 (CD35), CR3 (CD11b+CD18), and CR2 (CD21), respectively, which are expressed on myelomonocytic cells, lymphocytes and follicular dendritic cells (Holers, 2014). The binding of C3b or its metabolites to the correspondent cell receptors is crucial for cell-to-cell interaction within the innate and adaptive immune system and for removing apoptotic and necrotic cells (Ricklin *et al.,* 2010). Consecutive activation and binding of C3 leads to the generation of the C5 convertase which cleaves C5 into C5a and C5b. The membrane attack complex (MAC or C5b-9) is formed by C5b, C6, C7, C8 and

multiple C9 which insert into the cell membrane of target cells. Finally, MAC formation disrupts the phospholipid bilayer of the cell membrane, which causes massive calcium influx, loss of mitochondrial membrane potential and cell lysis. Collectively, the main three consequences of complement activation are (1) tagging target cells by C3b-metabolites for phagocytosis, (2) recruitment of inflammatory cells by C3a and C5a, and (3) initiation of MAC-mediated cell lysis (Figure 1; Tegla *et al.,* 2011).

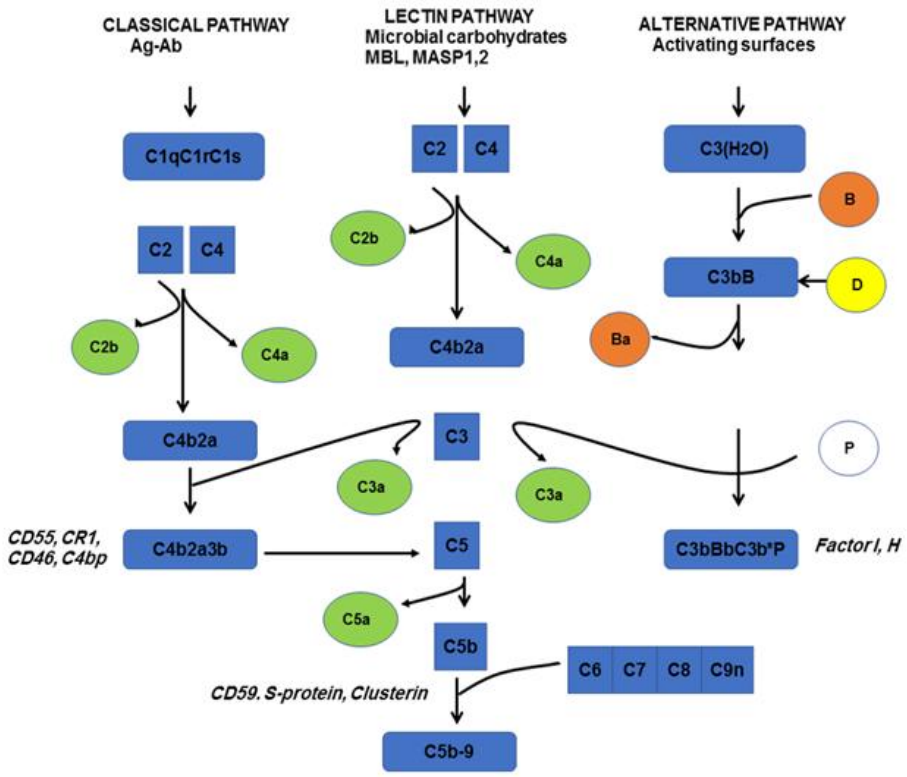


Figure 1: Complement activation pathways and assembly of the terminal pathway. The classical pathway is initiated by the binding of the C1 complex to antibody already bound to antigen, leading to the formation of the C4b2a enzyme complex (C3 convertase). The lectin pathway is activated by the binding of either MBL or ficolin and MASP 1, 2 and 3, respectively, to an array of mannose groups on the surface of bacterial cells and the generation of C3 convertase of the classical pathway. The alternative pathway is initiated by hydrolyzed C3 and factor B and the subsequent formation of the alternative pathway C3 convertase, C3bBb. Generation of the C3 convertase allows the formation of the C5 convertase, which initiates the formation of the C5b-9 terminal complement complex. The complement system is regulated at several levels: CD55, CR1, CD46, C4bp, and factors I and H regulate the activity of the C3- and C5-convertases. CD59 blocks the final assembly of MAC. S protein/vitronectin binds to C5b-7 and leads to the formation of a cytolytically inactive sC5b-9 complex. *This figure is reproduced from Tegla et al., 2011, with permission from Springer Nature.*

1.3.2. Complement regulation

Complement activation is tightly regulated at different points, either by fluid phase or membrane-bound complement regulatory proteins (mCRPs) (Tegla *et al.,* 2011; Holers, 2014) (Figure 1). C1-inhibitor belongs to family of serine protease inhibitors that inactivate proteins of several plasma cascades. In the classical pathway, C1-inhibitor regulates the auto-activation of C1 and inactivates the proteases C1r and C1s. In the lectin pathway, C1-inhibitor is an important regulator of the MBL-associated serine proteases (MASPs). It has been suggested that C1-inhibitor also regulates the AP where it binds non-covalently to C3b in a competition with complement factor B, a mechanism of inhibition that is completely different from the serpin function where C1-inhibitor binds covalently to the activated enzyme (Jiang *et al.,* 2001).

The AP is initiated continuously by low-grade hydrolysis of C3 which facilitates the covalent binding of C3b to any surface in a direct contact with the plasma upon formation of a fluid phase convertase C3b-H_2O-Bb (Fearon and Austen, 1975 a&b; Pangburn *et al.,* 1980). After formation of the C3 convertase, the target structures are effectively opsonised with C3b as a consequence of the amplification loop. Thus, activation of phagocytes and formation of MAC take place in parallel which can result in phagocytosis and cell lysis, respectively (Mueller-Eberhard, 1986). Regulation of the AP occurs at the C3b level by the plasma protein factors H (fH), Factor I (fI) and by three mMCRPs (CD35, CD46, and CD55) (Morgan and Meri, 1994; Liszewski *et al.,* 1996). Factor H regulates complement activation by competing with factor B for binding to C3b, by enhancing the dissociation of the C3 convertase, C3bBb and by acting as a cofactor for factor I in the proteolytic inactivation of C3b (Whaley and Ruddy, 1976; Weiler *et al.,* 1976; Fearon, 1978; Pangburn *et al.,* 1977).

Furthermore, complement regulatory proteins show multiple (e.g. decay-accelerating and membrane cofactor) activities. Regulators with decay-accelerating activity (C4-binding protein (C4bp), CR1/CD35, decay-accelerating factor (DAF/CD55), and factor H) disrupt the C3 convertase complex. However, those with cofactor activity (C4bp, CR1/CD35, MCP/CD46, and factor H) exert this function for factor I to cleave C3b and C4b into their inactive metabolites (iC3b and iC4b), respectively (Davies and Lachmann, 1993). CD59 interacts with C8 and C9 and inhibits the assembly of MAC (Davies and Lachmann,

1993). Accumulation of sublytic MAC on the cell membrane confers resistance to complement dependent cytotoxicity (CDC) and also induces the transduction of intracellular signals which induces cell proliferation (Tegla *et al.,* 2011). The anaphylatoxins C3a and C5a are rapidly inactivated by carboxypeptidases, particularly carboxypeptidase N (Matthews *et al.,* 2004).

1.4. Treatment approaches and development of resistance in tumors

1.4.1. Chemotherapy and resistance mechanisms

In cancer, different treatment approaches have been used including surgery, radiation therapy, chemotherapy, combination therapy, laser-based therapy and selective therapy (Longley and Johnston, 2005). Chemotherapy with one or more anti-cancer agent has been used as a part of standardized treatment regimen either with a curative intent or for prolongation of survival and reduction of the symptoms (palliative chemotherapy; Longley and Johnston, 2005).

Over the past decades a plethora of cytotoxic drugs, which selectively, but not exclusively, targets the proliferating cells were developed. These are consisting of different groups of reagents such as DNA alkylating agents, antimetabolites, intercalating agents and mitotic inhibitors (Luqmani, 2005). The antitumor potential of chemotherapeutic agents relies on six mechanisms: (1) DNA damage or inhibition of DNA replication, (2) inhibition of DNA and RNA synthesis, (3) interference with RNA transcription, (4) inhibition of protein synthesis, (5) interference with hormone homeostasis and (6) disruption of cellular microtubules via stabilisation or destabilisation (Kishi and Ueda, 2014). Novel strategies, based on molecular targeting of oncogenes, tumor suppressor genes and RNA interference (RNAi) were also developed (Nabholtz and Slamon, 2001).

Development of resistance to chemotherapeutic agents is a major problem, as it limits the effect of treatment. Drug-resistance is broadly classified into primary and acquired resistance (Lippert *et al.,* 2011; Meads *et al.,* 2009). During cancer invasion and metastasis, 90% of the failure in response to chemotherapeutic agents is due to the development of resistance. Drug-resistant cells develop remarkable features including modification of drug

transport, mutation of extracellular receptors, amplification and mutation of drug targets (Longley and Johnston, 2005).

Primary resistance is present prior to any given treatment, whereas acquired resistance develops after initial treatment. In cancer, acquired resistance is a particularly important problem, as tumor cells not only become resistant to the initially given drug but often develop cross-resistance to other drugs. Furthermore, resistant micrometastic tumor cells are responsible for the reduction of the effectiveness of chemotherapeutic agents in the adjuvant setting (Longley and Johnston, 2005).

Unfortunately, the majority of patients develop resistance at certain points of treatment. In the 1990s, nearly 50% of all cancer patients developed resistance against drugs (Pinedo and Giaccone, 1998). Approximately 20% of adults with acute lymphoblastic leukemia (ALL) present with a primary drug resistant phenotype (Testi *et al.,* 1992; Giona *et al.,* 1994; Thomas *et al.,* 1999; O'Connor *et al.,* 2011). Following surgery, 50–70% of cases with adenocarcinoma relapse with a chemoresistant phenotype (Castells *et al.,* 2012).

Several mechanisms of drug resistance are differentiated including drug inactivation, initiation of multi-drug resistance, cell death inhibition (apoptosis suppression), altered drug metabolism, epigenetic changes and modification of drug targets, enhancement of DNA repair and amplification of genes which cause chemotherapeutic drug resistance (Figure 2; Mansoori *et al.,* 2017). Multidrug resistance (MDR) is a complex phenomenon in which cancer cells become resistant to multiple drugs with different structures and distinct mechanisms of action (Gottesman, 1993; Gillet and Gottesman, 2010). Mechanisms of MDR in cancer include altered activity of certain enzyme systems such as glutathione S-transferase and topoisomerase which attenuates the efficacy of anti-cancer drugs (Lewis *et al.,* 1989; Seitz *et al.,* 2010).

Proteins of the BCL2 family play a fundamental role in apoptosis control. Changes in the expression level of these proteins are associated with malignancies and development of drug resistance (Reed, 1995). However, over-expression of adenosine triphosphate (ATP)-binding cassette (ABC) transporters like ABCB1 (P-glycoprotein, P-gp/MDR1), ABCCs [MDR-

associated proteins (MRPs)], and ABCG2 transporters (BMCRP/MXR/ABCP) is the most abundant MDR mechanism (Van Veen and Konings, 1998).

Cancer cells rely on energy derived from ATP hydrolysis and on protein transporters to efflux and significantly reduce the intracellular concentration of the anti-cancer drugs and hence attenuate their efficacy (Wang *et al.,* 2012).

Drug resistance mediated by ABC transporters such as P-glycoprotein (P-gp) is continued to be a major obstacle to effective cancer chemotherapy. Cancer cells derived from epithelial layers of colon, liver, adrenal gland, and pancreas are highly expressing P-gp transporters, which confers a broad spectrum of resistance to various anti-cancer agents, including anthracyclines, vinca alkaloids, etoposide and taxanes (de Bruijn *et al.,* 1986; Roninson *et al.,* 1986; Van der Bliek *et al.,* 1987; Gros *et al.,* 1988). Additional transporters of ABC such as ABCCs and ABCG2 are also mediators of MDR in cancer cells (Cole *et al.,* 1992; Van Veen and Konings, 1998; Guo *et al.,* 2003; Robert and Jarry, 2003).

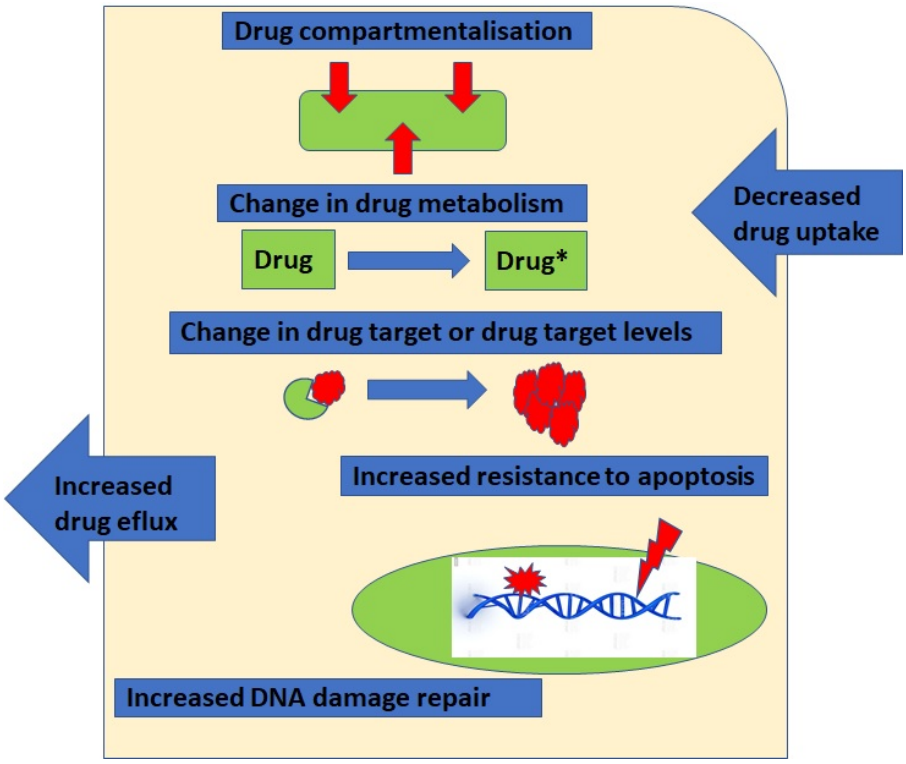


Figure 2: Mechanisms of drug resistance in the cancer cells. This includes inactivation of the drug, multi-drug resistance, cell death inhibition (apoptosis suppression), alteration of drug metabolism, epigenetic changes, drug targets modification, enhance d DNA-repair and target gene amplification. *This figure is reproduced from Mansoori et al., 2017 with permission from Advanced Pharmaceutical Bulletin.*

1.4.2. Monoclonal antibodies (mAbs)

Cancer-specific monoclonal antibodies (mAbs) are considered as one of the most successful targeted treatment strategies in oncology. Various surface antigens like: (1) growth and differentiation associated proteins, such as CD20, CD30, CD33 and CD52; (2) carcino-embryonic antigen (CEA); (3) epidermal growth factor receptor (EGFR); (4) receptor activator of nuclear factor kappa-B ligand (RANKL); (5) human epidermal growth factor receptor 2 (HER2); (5) vascular endothelial growth factor (VEGF); (6) VEGF receptor (VEGFR); (7) integrins (e.g. αVβ3 and α5β1); (8) fibroblast activation protein and (9) extracellular matrix metalloproteinase inducers have been targeted by mAbs (Hofmeister *et al.,* 2006; Hudis, 2007; Schliemann and Neri, 2007; Scott *et al.,* 2012). Several mAbs are commercially available for treatment of various malignancies. In haematological malignancies, rituximab and ofatumumab (RTX and OFA; anti-CD20) and alemtuzumab (ALM; anti-CD52) are widely used. In solid tumors, monoclonal antibodies such as trastuzumab (anti-HER2, herceptin) and cetuximab (anti-EGFR) were approved (Schliemann and Neri, 2007; Scott *et al.,* 2012).

Therapeutic mAbs exert direct and indirect anti-tumor effects. As direct mechanisms (Fab-mediated), mAbs induce anti-proliferative, pro-apoptotic signals or prevent binding of ligands (e.g. growth factors and cytokines) to their natural receptors. As indirect mechanisms (Fc-mediated), immune effector actions such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and complement-dependent cellular cytotoxicity (CDCC) are employed.

Target cells, opsonised with mAbs are subject to lysis either by CDC or CDCC. Recruitment of cytotoxic effector cells, such as natural killer (NK) cells, macrophages, and polymorphonuclear leukocytes (PMN) with Fc gamma receptors (FcγR) is essential for ADCC (Beers *et al.,* 2009). While ADCC and the Fab-mediated elimination of tumors are widely considered as effective mechanisms of mAb action, the role of complement in cancer immune response is still controversial (Beers *et al.,* 2009; Introna and Golay, 2009). Immunostimulatory mAbs efficiently activate T-lymphocytes by suppressing expression of CTLA-4 (Yang *et al.,* 2007). An additional set of mAbs induce vascular ablation and disrupt the interaction between stromal and cancer cells

which in turn inhibits the blood supply and supports tumor regression (Willett *et al.,* 2004).

In 1997, rituximab (RTX) was approved as the first mAb for cancer treatment (Wood, 2001). It is a chimeric anti-CD20 mAb composed of a murine variable region (Fab region) linked to a human (Fc region) reacting with CD20 on tumor cells. CD20 is a 35 kDa transmembrane protein, mainly located on pre-mature and mature B lymphocytes but not on stem or plasma cells (Lina Reslan *et al.,* 2009). RTX has become the standard treatment for B-cell malignancies including NHL (Armitage *et al.,* 1998). It achieved an impressive (48%) overall response rate in patients with relapsed low-grade NHL (McLaughlin *et al.,* 1998). In patients with chronic or small lymphocytic leukaemia, RTX showed an overall response rate of 58% with a 9% complete response rate (Hainsworth *et al.,* 2003). Similar success was reported when the drug was used to treat follicular lymphoma lymphoma (Hiddemann *et al.,* 2005), or diffused large B-cell lymphoma (DLBCL or DLBL; Coiffier *et al.,* 2002).

Development of new treatment strategies against breast cancer has focused on understanding the expression, regulation and function of critical signaling pathways involved in cancer initiation and progression which resulted in identification of breast cancer subsets with distinct biology (Perou *et al.,* 2000; Sorlie *et al.,* 2001; Sorlie *et al.,* 2003; Hu *et al.,* 2006) and development of targeted therapies. Notable examples are the successful use of hormonal therapy for women with hormone-sensitive tumors and anti-HER2-based therapy for women with HER2-overexpressing tumors (Slamon *et al.,* 2001).

Trastuzumab (herceptin) is a humanized IgG1 *kappa* light chain mAb in which the complementary-determining regions of a HER2-specific mouse mAb were joined to human antibody framework regions through genetic engineering (Carter *et al.,* 1992; Fendly *et al.,* 1990). It has been approved by the U.S. Food and Drug Administration (FDA) for treatment of HER2-overexpressing breast cancer in adjuvant and metastatic settings (Cobleigh *et al.,* 1999; Slamon *et al.,* 2001; Romond *et al.,* 2005). The importance of the anti-tumor effect of trastuzumab-induced ADCC was shown in several xenograft models (Clynes *et al.,* 2000; Spiridon *et al.,* 2004; Barok *et al.,* 2007). However, the clinical relevance of complement activation mediated by trastuzumab is less clear. Trastuzumab has been shown to fix complement and cause destruction of the

HER2-positive cell line BT474 *in vitro* (Spiridon *et al.,* 2004). In other studies, trastuzumab was only able to induce a minor effect by CDC (*Drebin et al., 1988;* Yu *et* al., 1999; Prang *et al., 2005*; Mamidi *et al., 2013*).

1.4.3. Response to combined mAbs/chemotherapeutic anti-cancer regimens

Although mAbs improve the response of patients suffering from certain types of cancers, there is no evidence that they can positively modify curability of conventional therapy-resistant patients (Prang et al., 2005; Mamidi et al., 2013). Combination regimens of RTX with cyclophosphamide, hydroxydaunomycin, oncovin, prednisone (CHOP) improved response rate, freedom from progression and overall survival of patients with DLBCL who were already curable with CHOP alone (Coiffier *et al.,* 2002). However, patients resistant to RTX-containing chemotherapy or those who relapsed after treatment showed a very low survival rate. Likewise, in solid tumors mAbs in combination regimens could not improve curability of patients resistant to conventional therapy (Bhutani and Vaishampayan, 2013).

Whether administered as a single agent or in combination regimens, the therapeutic efficacy of mAbs is often restricted by resistance mechanisms. Among possible mechanisms of resistance to RTX are down-regulation or loss of expression of CD20, formation of soluble CD20 molecules or inhibition of ADCC and CDC (Smith, 2003; Taylor and Lindorfer, 2010). Bail *et al.,* (2010) demonstrated that the reduced sensitivity of B cell lymphomas to RTX-mediated CDC was in part due to down-regulation of CD20 expression. In contrast, enhanced expression of CD20 by B cell lymphomas in response to the farnesyl transferase inhibitor bryostatin-1 or histone deacetylase inhibitors increased the RTX -induced cytotoxic activity (Wojciechowski *et al.,* 2005; Shimizu *et al.,* 2010; Winiarska *et al.,* 2012). Likewise, enhanced expression of CD20 on DLBCL cells in response to gemcitabine augmented their sensitivity to rituximab-induced CDC (Hayashi *et al.,* 2016). It has been reported that drug resistant cancer cells exert either enhanced resistance or enhanced susceptibility to CDC (Weisburg *et al.,* 1996; Bomstein and Fishelson, 1997; Odening *et al.,* 2009). This is mainly attributed to expression of P-gp and abnormal expression of complement regulators (Bomstein and Fishelson, 1997;

Odening *et al.,* 2009). Similarly, various chemotherapeutic drugs effectively inhibited NK cells-mediated tumor cell killing and significantly impaired the efficacy of immunotherapy (Markasz *et al.,* 2007).

1.5. Tumor resistance to complement-dependent cytotoxicity (CDC)

Under normal conditions, complement is tightly regulated by circulating and membrane-bound complement regulators (mCRP) to avoid complement-mediated cell destruction. However, in certain tumours, the cells tend to express higher levels of mCRPs than normal cells (Niehans *et al.,* 1996; Simpson *et al.,* 1997; Varsano *et al.,* 1998a; Li *et al.,* 2001; Fishelson and Kirschfink, 2019). Colorectal, lung, prostate and breast cancers highly express at least one of the mCRPs (Koretz *et al.,* 1992; Hosch *et al*., 2001; Fishelson *et al.,* 2003; Liu *et al.,* 2005; Loberg *et al.,* 2006; Macor &Tedesco, 2007; Gancz and Fishelson, 2009; Zhao *et al.,* 2009).

High expression of CD55 by B-lymphocytes reduced their sensitivity to RTX-mediated CDC (Terui *et al.,* 2006). Up-regulation of CD59 in NHL and CLL malignancies was considered as a marker for sensitivity to RTX and OFA (Bjorge *et al*., 1997; Jarvis *et al.,* 1997; Juhl *et al.,* 1997; Coral *et al.,* 2000; Fonsatti *et al.,* 2000; Golay *et al.,* 2000; Harjunpaa *et al.,* 2000; Golay *et al.,* 2001; Ziller *et al.,* 2005; Bjorge *et al.,* 2006; Takei *et al.,* 2006; Macor *et al.,* 2007a; Hu *et al*., 2011). RTX-mediated CDC linearly correlated with the expression of CD20 in freshly isolated B-CLL, mantle cell lymphoma and prolymorphocytic leukemia cells. Moreover, functional inhibition of CD55 and CD59 increased sensitivity of these tumor cells to both RTX and complement (Golay *et al.,* 2001). Primary CLL and CD20-positive Raji cells showed reduced sensitivity to RTX-induced CDC, whereas the same cells were more susceptible to OFA-induced CDC (Mamidi *et al.,* 2015). Primary CLL cells were more sensitive to ALM-induced CDC (Mamidi *et al.,* 2015). The combined inhibition of mCRPs on Raji and CLL cells enhanced their sensitivity to RTX and OFA-induced CDC (Mamidi *et al.,* 2015). Similarly, inhibition of mCRPs on CD52-positive, ALL and REH cells augmented their sensitivity to ALM-induced CDC (Mamidi *et al.,* 2015).

Reduced sensitivity of breast carcinoma cell lines to CDC induced by MT201 (epithelial cell adhesion molecule (Ep-CAM)-specific mAb) and

trastuzumab correlated with the expression of complement resistance factors (Prang *et al.,* 2005). The inefficient ability of trastuzumab to induce CDCC or CDC of HER2-positive glioblastoma cell lines (A172 and U251MG) was due to elevated expression of CD55 and CD59 (Mineo *et al.,* 2004). Expression of CD55 and CD59 by non-small cells lung carcinom closely correlated with the histological type, prognosis and the effect of the pre-operational adjuvant chemotherapy. Functional inhibition of mCRPs increased killing of two human lung carcinoma cell lines by the trastuzumab-induced CDC (Zhao *et al.,* 2009).

Phosphorylation of intracellular proteins by the protein kinase C (PKC) (Jurianz *et al.,* 1999; Kraus and Fishelson, 2000), of the extracellularly regulated protein kinase (ERK) (Kraus *et al.,* 2001) and increased protein synthesis (Ohanian *et al.,* 1981) entail complement resistance mechanisms. The possible mode of protection conferred by PKC and ERK is that they take part in the process of MAC removal by vesiculation or endocytosis (Carney *et al.,* 1985; Morgan *et al.,* 1987).

Sublytic doses of antibody and complement or treatment with ionomycin, A23187, cAMP, forskolin or phorbol myristate acetate enhanced resistance of erythroleukaemic K562 cells to complement-mediated damage within several minutes (Reiter *et al.,* 1995). The protective effect of the sublytic C5b-9 complement complex doses relies on initiating signalling processes resulting in calcium ion influx, PKC and ERK activation and protein synthesis (Reiter *et al.*, 1992; Kraus and Fishelson, 2000; Kraus *et al.,* 2001). Similarly, the pore-formers perforin, streptolysin O and mellitin enhanced resistance of the K562 cells to complement (Reiter *et al.,* 1995).

Blocking the activity of complement fH has been suggested as a powerful strategy to increase the response of resistant tumors to RTX-induced CDC (Di Gaetano *et al.*, 2003; Van Meerten *et al.,* 2006). Horl *et al.,* (2013) reported that the short-consensus repeat 18–20 (hSCR18–20) of human recombinant complement fH was able to interfere with binding of fH to CLL cells and enhanced their sensitivity to RTX-induced CDC. Recombinant antibody produced against fH was able not only to activate complement to release anaphylatoxins and promote CDC of tumor cell lines but also to inhibit tumor growth *in vivo* (Bushey *et al.,* 2016).

Additional strategies and approaches to increase the sensitivity of cancer cells to complement attack are in progress. Engineering of cancer cells to express the heterologous antigen α-Galactose (α-Gal) efficiently increased their sensitivity to lysis by activating the complement cascade. Wu *et al.* (2014) reported that human colorectal adenocarcinoma cell lines (LoVo and SW620) transfected with α-Gal were highly sensitive to complement attack compared to CD55-highly expressing Ls-1740T cells.

1.6. Rational of the present work

Although many drugs and mAbs are in clinical use to treat cancer, a complete therapeutic response can be achieved in only few patients. This can often be attributed to the development of resistance to the used therapies. Despite increasing evidence that cancer drugs confer resistance to CDC, ADCC, and CDCC, little is still known about the interaction between chemo- and immuno-therapy within combined treatment regimens. This encouraged us to further study the impact of chemotherapy on antibody-based complement-mediated tumor cell killing.

1.6.1. Aim of the study

In an in *vitro* study, we aim to investigate the possible implication of known anti-cancer drugs on complement-mediated tumor cell lysis and/or opsonisation with the C3 fragment iC3b and the potential involvement of complement regulatory proteins.

1.6.2. Specific objectives are:

a. to test the effect of treating tumor cell lines with anti-cancer chemotherapies on their susceptibility to antibody-mediated opsonisation and CDC.
b. to study the dose dependent effect of such chemotherapeutics on the expression profile of complement regulators.

1.6.3. Plan of the work

a. Raji, SKBR-3 and BT474 cells are used as models for hematologic and breast cancer.
b. Various concentrations of the chemotherapeutics doxorubicin, taxol, bortezomib and fludarabine are used at two different time intervals to pre-treat the tumor cells.
c. Expression of membrane complement regulators (mCRP) on treated and untreated cells will be tested.
d. Response of treated and untreated cells to antibody-based complement-mediated cell killing (CDC) in absence and in presence of neutralizing anti-mCRP Abs will be studied.
e. Binding of exogenously added complement regulator fH to treated and untreated cell membranes will be analyzed.
f. Tagging of treated and untreated cells with the opsonine iC3b upon complement activation will be investigated.
g. Secretion of soluble complement regulators like C1-inhibitors, fH and fI from treated and untreated cells will be analyzed.

Chapter 2– Materials & Methods

2.1 Materials

2.1.1 Equipment

Autoclave	Systec 5075 EL, Wettenberg, Germany
CO_2 incubator	Heraeus B 5060 EK/CO_2, Hanau, Germany
Cell counter	CASY TT, Roche Innovatis, Mannheim, Germany
Centrifuges	1. ALC 4236, Wiesloch, Germany 2. Hettich Rotina 48R, Tuttlingen, Germany 3. Eppendorf, 5415R, Hamburg, Germany
Microplate reader for ELISA	Sunrise Tecan, Crailsheim, Germany
Flowcytometer	FACS LSR II, Becton Dickinson (BD), Heidelberg, Germany
Heating &magnetic stirrer plate	Heidolph MR 3001K, Neolab Migge, Heidelberg, Germany
Inverted microscope	Carl Zeiss ID 03, Jena, Germany
Laboratory pH meter	pH -meter 766 Calimatic, KNICK, Berlin, Germany
Light microscope	Carl Zeiss 4730 11-9901, Jena, Germany
Sterile bank	UVF 618S-BDK, Sonnenbuehl-Genkingen, Germany
Vortex-mixer	HeidolphElectrical/Electronic Manufacturing, Kelheim, Germany
Water path	Julabo SW22 E-7104, Neo-Lab Migge, Heidelberg, Germany
Vacuum-pump	Vacuubrand, Wertheim, Germany
γ–counter	Automated Wizard^2 2470, Perkin Elmer's MA, USA

2.1.2 Non-disposable materials

Glass tubes	Central store, Heidelberg University, Germany
Neubauer counting chamber	Brand, Wertheim, Germany
Micro vials (2.5ml; 5ml)	Neo-Lab Migge, Heidelberg, Germany

2.1.3. Disposable materials

Six well plates	Nunc Int., Roskilde, Denmark
Ninety-six well plates (flat, U-shape or V-shape bottom)	Nunc Int., Roskilde, Denmark
Counting tubes	Ritter, Schwabmuenchen, Germany
Cell culture flasks ($25cm^2$ &$75cm^2$)	Nunclon^{TM} , Nunc Int., Roskilde, Denmark
Freezing tubes	Nunclon^{TM} Nunc Int., Roskilde, Denmark
FACS-tubes	BD, Heidelberg, Germany
Falcon tubes (15ml &50ml)	Greiner bio-one, Frickenhausen, Germany
Micropipette tips	Eppendorf, Hamburg, Germany
Serological pipettes, sterile (5ml &10ml)	Sarstedt, Nuembrecht, Germany
Micropipettes (2µl -1000µl)	Eppendorf, Hamburg, Germany
Minisart syringe filter (0.2µm)	Sartorius, Goettingen, Germany
Serological pipette sterile (25ml)	Greiner Bio-one, Frickenhausen, Germany
Nalgene rapid flow 75mm filter unit (500ml -1000ml)	Thermo-Scientific, Germany
Pasteur-pipettes	Hilgenberg, Malsfeld, Germany
Weighting papers	A. Hartenstein, Wuerzburg, Germany

2.1.4. Reagents

Aqua ad injection	B. Braun Melsungen, Germany
BSA (Bovines Serum Albumin)	SERVA, Heidelberg, Germany
DMSO (Di-Methyl Sulfoxide)	Sigma-Aldrich, St. Louis, MO, USA
EDTA	Roth, Karlsruhe, Germany
Ethanol (70% ig)	Central store, Heidelberg University, Germany
FCS (Fetal Calf Serum)	Invitrogen, Karlsruhe, Germany
H_2O_2 (Hydrogen peroxide)	Sigma-Aldrich, St. Louis, MO, USA
Sodium azide (NaN_3)	Merck, Darmstadt, Germany
Paraformaldehyde (PFA)	Central store, Heidelberg University, Germany
Hydrochloric acid (HCL)	Mallinckrodt Baker, Deventer, Netherlands
Triton® X-100	MERCK, Darmstadt, Germany
Trypsin-EDTA	PAA Laboratories, Coelbe, Germany
Trypan blue solution 0.4%	Sigma-Aldrich, St. Louis, MO, USA
ABTS (2,2'-Azino-Bis-3 Ethyl benzo thiazoline-6-Sulphonic acid)	Sigma-Aldrich, St. Louis, MO, USA

2.1.5. Buffers and cell culture media

Ca^{2+}/Mg^{2+} - stock solution	0.15M $CaCl_2$; 1M $MgCl_2$
EDTA- stock solution	37.2g EDTA, 6ml NaOH (32%) and 1L distilled water, the pH was adjusted to 7.5 with NaOH (32%)
Calibration solutions for the pH meter	pH 4; pH 7; pH 9.2. Mettler-Toledo, Urdorf, Switzerland
FACS buffer	1x PBS; 1% BSA; 0.1% NaN_3
Fixation buffer	1 x PBS; 1% PFA.

HEPES buffer	Life Technologies, Darmstadt, Germany
PBS	Life Technologies, Darmstadt, Germany
RPMI 1640	Life Technologies, Darmstadt, Germany
McCoy's 5A Modified Medium	PAN Biotech, Aidenbach, Germany
Test medium	0.03% 2M $MgCl_2$; 0.1% BSA in culture medium without Fetal Calf Serum (FCS)
Veronal Buffer Saline (VBS) stock solution	5mM Sodium Barbital (pH 7.4); 150mM $NaCl_2$.
VBS working solution	VBS-stock buffer was diluted with distilled water (1:5); 0.15mM $CaCl_2$; 1mM $MgCl_2$; 0.1% BSA
Washing buffer	1 x PBS, 2mM EDTA.
ELISA buffers	1. Washing buffer: PBS; 0.1% Tween-20 (pH 7.4) 2. Coating buffer: 50mM $Na2CO_3$ (pH 9.6) 3. Antibody dilution buffer: PBS; 0.2% Tween-20 (pH 7.4) 4. Samples dilution buffer: PBS; 0.2% Tween-20; 10mM EDTA (pH 7.2) 5. Substrate buffer: ABTS 2mg/ml Citrate-Phosphate buffer (pH 4.6)
ELISA stopping buffer	Oxalic acid (0.2M); Grüssing Analytica, Filsum, Germany

2.1.6. Test kits

FITC-labeled Annexin V for apoptosis detection kit I	BD, Heidelberg, Germany.
^{51}Cr	Hartmann-Analytic, Braunschweig, Germany

2.1.7. Chemotherapeutic agents

Doxorubicin (2mg/ml)	(Adriamycin); Central pharmacy, Heidelberg University Hospital
Fludarabine (10mg/ml)	(Fludara); Central pharmacy, Heidelberg University Hospital
Bortezomib (2.5mg/ml)	(Velcade); Central pharmacy, Heidelberg University Hospital
Paclitaxel (PTX; 6mg/ml)	(Taxol); Bristol Arzneimittel, Munich, Germany

2.1.8. Antibodies, ELISA controls and standards

FITC-labeled f(ab')2 gt α ms IgG	DAKO-BIOZOL, Eching, Germany
Alexa fluor 488-labeled rb α gt IgG	Life Technologies, Darmstadt, Germany
ms α iC3b	Quidel (A209); Kornwestheim, Germany
ms α CD46	IgG1, clone GB24; Dr. J. Atkinson, Washington University, St. Louis, MO, USA
ms α CD55	IgG1, clone Bric 216; International Blood Group Reference Laboratory, IBGRL, Birmingham, England
ms α CD59	IgG2b, clone Bric 229; International Blood Group Reference Laboratory, IBGRL, Birmingham, England
ms IgG1 isotype control	15H6; Southern Biotech, Birmingham, AL, USA
ms IgG2b isotype control	A-1; Southern Biotech, Birmingham, AL, USA
Polyclonal rabbit antiserum against tumour cells	In-house produced, Institute of Immunology, Heidelberg, Germany (Odening *et al.,* 2009)

Rituximab (10mg/ml)	Rituxan®, Central pharmacy, Heidelberg University Hospital
ELISA, factor I (fI) -antibodies 1. gt α human fI 2. ms α human fI 3. F(ab')2 gt α ms IgG (H+L)-PO	 Quidel (A313); Kornwestheim, Germany Quidel (A247); Kornwestheim, Germany Dianova 115-036-062), Hamburg, Germany
Factor I control	Control sera SEKO14 and SEPO4 from Diagnostic lab, Institute of Immunology, Heidelberg University, Germany
Factor I standard (30µg/ml)	Calbiochem, Bad Soden, Germany
ELISA, factor H (fH) -antibodies 1. gt α human fH 2. ms α human fH 3. F(ab')2 gt α ms IgG (H+L)-PO	 Quidel (A312); Kornwestheim, Germany Serotec (MCA 509, OX 24 S/N); Biorad, Puchheim, Germany Dianova (115-036-062), Hamburg, Germany
Factor H standard (1.05mg/ml)	Advanced Research Technologies, San Diego, CA, USA
Factor H controls	Control serum SEKO 10 and Kons. 128694 from the Diagnostic lab, Institute of Immunology, Heidelberg, Germany
ELISA, C1-Inhibitor (C1-Inh) antibodies 1. rb polyclonal α human C1-Inh 2. gt polyclonal α human C1-Inh 3. rb polyclonal α gt IgG (H+L)-PO	 AK 133; DAKO-BIOZOL, Eching, Germany AK 47; ATAB, Germany Dianova (305-036-45), Hamburg, Germany

C1-Inh standard (10.9mg/ml):	GSL; Behring, Germany
C1-Inh controls	Control sera SEPO4 and SEKO14 from Diagnostic lab, Institute of Immunology, Heidelberg, Germany
gt polyconal α human fH	MERCK-Biosciences, Darmstadt, Germany

2.1.9 Normal human serum (NHS) as a source of complement

Active serum	A pool of sera from healthy blood donors, sterile filtrated and stored at -70ºC.
Inactive (i.a.) serum	The same pool of sera was complement-inactivated by heating at 56ºC for 30min
C8 depleted serum	Human serum deficient in C8, Complement Technology Inc., Texas, USA

2.1.10 Tumor cell lines

Cell line	**Adherent/ Suspension**	**Cell type**	**Origin**	**Medium**	**DSMZ -Nr.**
BT474	Adherent	Mammary gland; ductal carcinoma	60 years adult with invasive breast cancer	RPMI-1640	ACC-64
SKBR3	Adherent	Breast carcinoma	Established from the pleural effusion of a 43-year-old Caucasian woman with breast carcinoma in 1970	McCoy´s - 5a	ACC-736
Raji	Suspension	Burkitt-lymphoma	Established from the left maxilla of a 12-year-old African boy with Burkitt lymphoma in 1963.	RPMI-1640	ACC-319

2.2 Methods

2.2.1 Cell culture

SKBR3 cells were cultured in McCoy's 5a (90% McCoy´s 5a + 10% h.i. FCS), BT474 and Raji cells were maintained in RPMI 1640 media (90% RPMI 1640 + 10% h.i. FCS + 10µg/ml human insulin + 2mM L-glutamine). For all cell lines, the media were supplemented with 10% FCS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. The cells were grown under standard conditions (humidified atmosphere, 5% CO_2 at 37ºC) and passaged two or three times a week to keep the culture in logarithmical growth. For propagation and seeding, adherent cells were washed with PBS, trypsinized in 0.25% Trypsin/EDTA and the cell pellets were collected by centrifugation at 200×g for 5min. Cell count was determined, followed by re-suspending the cells into desired cell density according to the experimental needs (Mamidi *et al.,* 2013). Cells growing in suspension were collected by centrifugation as mentioned above, followed by cell counting in appropriate fresh culture medium. Finally, cell densities were adjusted according to the experimental needs (Mamidi *et al.,* 2015).

2.2.2 Cell lines storage

Storing and reseeding of frozen cells were carried out according to DSMZ regulations. Briefly, tumor cells were harvested from cell culture flasks and re-suspended in freezing medium consisting of 70% fresh culture medium (appropriate medium for each cell lines), 20% FCS, and 10% DMSO. The cell density was adjusted at 1-1.5×10^6cells/ml and 1ml of cell suspension was immediately transferred into a sterile cryo-vial and stored at -80ºC for short term or in liquid nitrogen for long term storage. Frozen cells were thawed directly in water bath at 37ºC and immediately mixed in 15ml falcon tubes with 10ml fresh cell culture medium containing 10% FCS. The cells were collected by centrifugation at 200×g for 5min, the supernatant was discarded and the cell pellets were re-suspended in an appropriate volume of culture medium.

2.2.3 Cell treatment

Anti-cancer agents (Doxorubicin 2mg/ml; Fludarabine 10mg/ml; Paclitaxel (Taxol) 6mg/ml; Bortezomib 2.5mg/ml) were stored at 4ºC as stock solution until use. Working drug concentrations were freshly prepared in appropriate cell culture media and precisely mixed before use. In all further experiments, the term un-treated is referring to tumor cells grown in absence of anti-cancer agents. The term 'treated' refers to tumor cells grown in presence of one of the anti-cancer agents at indicated time interval.

2.2.4 Trypan blue dye exclusion assay

The cells were seeded in 6-well cell culture plates at $8x10^5$ cells/well and adherent cells were allowed to grow 24h before treatment. Cells were then pre-treated with different concentrations of anti-cancer drugs: taxol (0, 5, 10 and 20nM); doxorubicin (0, 2, 5 and 10µM); bortezomib (0, 5, 10 and 20nM) for 48h under standard growth conditions (humidified atmosphere, 5% CO2 and 37ºC). For Raji cells, cell density was adjusted to $8x10^5$cells/ml followed by immediate treatment with above mentioned bortezomib concentrations for 48h. Raji cells were pretreated with fludarabine (0, 1, 3 and 5µM) at 48h under standard growth conditions. Working drug concentrations were used for doxorubicin (Pilco-Ferreto and Calaf, 2016), taxol (Liebmann *et al.,* 1993), bortezomib (Di Raimondo *et al.,* 1995) and fludarabine (Bil *et al.,* 2010) as previously recommended. The trypan blue dye exclusion assay was used to determine the number of viable cells in a cell suspension (Strober *et al.,* 2001). Briefly, 50µL of the cell suspension were added into a cryo-vial, mixed with an equal volume of 0.4% trypan blue dye and incubated for at least 3min at room temperature. With the coverslip, one side of a hemacytometer counter was filled with the cell suspension and, using a conventional light microscope, coloured (death) cells were counted and the percentage of viable cells was calculated. As we here analyse the impact of anti-cancer drugs on antibody mediated cancer cell killing or opsonisation, tolerable, non-toxic concentrations of used anti-cancer drugs are indispensable to use for further analysis in this study.

2.2.5 Analysis of mCRP expression

$8x10^5$ cells/well of each tumor cell line were prepared in duplicates and seeded into six well cell culture plates. Adherent cells as well as growing cells in a suspension were handled as previously described. Levels of mCRPs were analyzed by flow cytometry assay according to Mamidi *et al,* 2015. Briefly, collected tumor cells were re-suspended and distributed at $1x10^5$/100µL FACS buffer (1% BSA, 0.1% NaN_3 in PBS)/well in V-shape bottom 96 well plates. The cells were washed twice in FACS buffer by centrifugation at 150×g each for 5min and 4°C. Different sets of the cells were incubated at 4°C for 30min with either ms α human primary antibody against CD46, CD55 and CD59 or with ms IgG1 isotype control (10µg/ml). The cells were then washed twice, followed by centrifugation for 5min at 4°C in FACS buffer. The cell pellet in each well was re-suspended in 100µl FACS buffer containing FITC-conjugated f(ab')2 goat α mouse IgG antibody (1:50) and incubated in the dark for 30min at 4°C. Finally, the cells were washed three times by centrifugation in FACS buffer 5min at 4°C and fixed in 100µl fixing buffer (4% paraformaldehyde in PBS). Stained cells were analysed using FACS LSRII. For each cell line, an appropriate voltage was adjusted on a sample from untreated cells. For each mAb used an appropriate isotype control was included for gating to exclude the background due to non-specific staining. The gated cells were analysed and the data were presented in percentage (mean values ± SD) of the givin tumor cells with positive mCRP expression. Untreated cells were taken as a reference and all experiments were performed 3 times with three biological replicates for each variable.

2.2.6 Analysis of fH binding to cancer cells

Tumor cells ($8x10^5$) cells/well were cultured in duplicates in cell culture medium in presence or absence of anticancer drugs as previously mentioned. At 48h, cells were collected as described above, washed once by centrifugation in PBS for 5min at room temperature and re-suspended in 1x PBS containing 1% BSA (PBS-BSA). The cells were further incubated with NHS diluted 1:4 in PBS-BSA –buffer, for 20min at 37°C. Binding of fH to tumor cells was detected using goat anti-human fH polyclonal antibody (1:500) in PBS-BSA and Alexa Fluor 488-labeled rabbit anti-goat IgG (1:800) in PBS-BSA. Goat pre-immune

sera were used as a negative control. 1×10^5 cells in three independent experiments with each 3 biological replicates at each drug concentration were analyzed by FACS LSRII as mentioned above. The data are presented as percentage (mean values ± SD) of gated tumor cells with positive fH binding. Untreated cells were taken as reference and all experiments were performed 3 times and three biological replicates for each variable were analysed.

2.2.7 Analysis of cell lysis by CDC

Complement-dependent lysis of tumor cells was analysed by radioactive ^{51}chromium (^{51}Cr) release assay as described by Mamidi *et al.,* (2015). Briefly, untreated and pre-treated tumor cells ($1x10^6$) were labeled in 100µl complete growth medium (appropriate medium for each cell lines with 10% FCS) with 100µCi ^{51}Cr for 2h at 37°C. Labeled cells were washed three times in assay medium (0.6mM $MgCl_2$ and 0.1% BSA in FCS-free RPMI or McCoy's 5a) and the cell count was adjusted at $2x10^5$ cells/ml in assay medium. The cell suspension ($1x10^4$/50µl) was distributed in U-shape bottom 96 well plates and incubated with complement activating antibody. For Raji cells RTX (10µg/ml) was used. To achieve optimal CDC and because of lack of broad reacting and CDC-inducing mAbs against breast cancer cell lines, polyclonal rabbit anti-tumour antibodies (1:50) from previously immunized rabbits (described in detail in Odening *et al.*, 2009) were used. In a set of experiments, mCRP specific non-complement activating neutralizing antibodies anti-CD46 (clone GB-24), anti-CD55 (clone Bric 110), and anti-CD59 (clone Bric 229) were applied at a concentration of 2µg/ml for 20min to inhibit the respective inhibitors. Normal human serum (NHS) (1:10) in assay medium were used as complement source for 60min at 37°C. Heat inactivated (i.a.) serum served as control. To determine the spontaneous ^{51}Cr release, NHS was replaced by test medium in few wells. Maximum ^{51}Cr release was determined by adding 1% Triton® X-100 into control wells. Finally, the plates were centrifuged at 150 × g for 5min and radioactivity in supernatants was measured in a γ-counter. The data are presented as the mean valus ± SD of lysed cells (in %). Untreated cells were taken as a reference and all experiments were performed 3 times with three biological replicates for each variable. Specific lysis of each sample was calculated

according to the following formula ((test release – spontaneous release)/ (maximum release – spontaneous release)) × 100.

2.2.8. Analysis of cell opsonisation with iC3b

Opsonisation with iC3b of untreated and drug-treated tumor cells in response to anticancer drugs was analysed by flow cytometry as described by Mamidi *et al.* 2013. Briefly, tumor cells ($1x10^5$/100µl) were re-suspended in VBS-buffer (5mM Sodium Barbital (pH= 7.4), 0.15mM $CaCl_2$, 1mM $MgCl_2$, 150mM $NaCl_2$; 0.1% BSA). In order to induce complement activation, cells were pre-incubated with either RTX (10µg/ml) final concentration or polyclonal rabbit anti-tumor antibodies (1:50) for 30min at 37°C. C8-depleted or heat inactivated human serum in VBS-buffer were added to avoid terminal MAC formation and further incubated for 20min at 37°C. The cells were washed twice with FACS buffer each for 5min at 4°C and iC3b opsonisation was quantified by applying (1) primary antibody: mouse monoclonal anti-iC3b/IgG (10µg/ml) final concentration for 30min at 37°C; (2) secondary antibody: FITC labeled goat anti-mouse IgG (1:50) for 30min at 37°C. Finally, cells were washed twice in FACS-buffer for 5min at 4°C. The data are presented in percentage (mean values ± SD) of gated tumor cells positive for iC3b staining. Untreated tumor cells were taken as a reference and all experiments were performed 3 times and three biological replicates for each variable were analysed.

2.2.9. Analysis of soluble complement inhibitor secretion (C1-inhibitor, factor H and factor I) by cancer cells

Levels of factor H (fH), factor I (fI) and C1-inhibitor (C1-Inh) in the collected cell culture supernatants of untreated and drug-treated cells were measured by enzyme-linked immunosorbent assay as described by Mamidi et al 2013. In brief, 96-well microtitre plates with flat-shape bottom were coated with the respective specific antibodies in 50mM Na2CO3/NaHCO3, pH 9·6 for 16h at 4°C [fH, (gt α human fH (1:1000); fI, (gt α human fI (1:300); C1-Inh, (rb polyclonal α human C1-Inh (1:300)]. After washing the plates three times in ELISA-washing buffer PBS; 0.1% Tween-20 pH 7.4, the remaining unspecific binding sites were blocked with 1% BSA in PBS, 100µl/well from each cell culture supernatant was added in triplicate for 1h at room temperature. The plates were washed three times and incubated with the respective detection

antibodies [fH, (ms α human fH (1:400); fI, (ms α human fI (1:1000); C1-Inh, (gt polyclonal α human C1-Inh (1:2000)] for 1 h at room temperature followed by peroxidase-conjugated secondary antibody f(ab')$_2$ rabbit anti-goat IgG (1:5000) or goat anti-mouse IgG (1:2000 and/or 1:1000), respectively, for 1h at room temperature. The assay was developed using ABTS 2mg/ml Citrate-Phosphate-buffer (pH 4.6)/H_2O_2 as a substrate. After terminating the reaction with 0.2M oxalic acid as stop solution, the microtitre plates were analysed at 405nm, with reference filter at λ max: 492nm on an ELISA plate reader. Purified C1-Inh, fH and fI were used as standards.

2.2.10. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons were performed using either Two-way ANOVA, followed by Bonferroni post hoc test or by using One-way ANOVA, followed by Dunn's Multiple Comparison test. The results are presented as mean values ± SD of triplicates of three independent experiments. For all studied parameters, untreated tumor cells were used as reference and the effects of tested drugs on these parameters were analysed at selected time intervals for each drug concentration. The differences between data sets were considered significant at $p < 0.05$.

Chapter 3 – Results

3.1. Viability of different cancer cell lines upon treatment with different chemotherapeutics at 48h

To allow interpretation of complement-mediated cytotoxicity (CDC) versus drug-induced cell killing, viability of tumor cells from different lines upon treatment with respective drugs was analysed at 48h in a dose dependent manner. The influence of anti-cancer drugs on the viability of cancer cells was almost neglectable at 24h, whereas a measurable influence was observed at 48h (Table 1 and Figure 3).

Untreated BT474 cells showed a viability of 89.1±2% at 48h. Doxorubicin-treated cells showed 80.2±2.8%, 72.4±2.1% and 69.4±2.2% viability at 2µM, 5µM and 10µM drug concentration, respectively. Upon treatment with taxol these cells had a viability of 83.2±3.7%, 72.9±3.1% and 70.7±3.2% at 5nM, 10nM and 20nM, respectively. Viability of bortezomib-treated cells was 82.7±3.7%, 79.2±3.7% and 69.7±2.6% at 5nM, 10nM and 20nM taxol, respectively (Table 3 & Figure 3a, c & e).

Untreated SKBR-3 cells showed a viability of 88.6±3.5% at 48h. Upon treatment with doxorubicin viability was 79.1±1.8%, 74.5±3.5% and 69.4±2.1% at 2µM, 5µM and 10µM, respectively (Table 1 & Figure 3a & b). Taxol-treated cells showed 81.4±1.7%, 75.8±4.5% and 71.1±1.5% viability at 5nM, 10nM and 20nM drug concentration, respectively (Table 1 & Figure 3c & d). Viability of bortezomib-treated cells was 75.4±3.9%, 68.8±1.4% and 63.7±3.5% at 5nM, 10nM and 20nM, respectively (Table 3 & Figure 3b, d & f).

Untreated Raji cells showed 91.2±1.8% viability at 48h. Upon treatment with bortezomib a viability of 83.7±3.9%, 74.8±3.2% and 66.0±5.7% was recorded at 5nM, 10nM and 20nM bortezomib concentration, respectively (Table 1 & Figure 3e, f & g). Fludarabine-treated Raji cells showed 81.2±1.9%, 73.5±4.2% and 70.0±3.1% viabilty at 1µM, 3µM and 5µM drug concentration, respectively (Table 3 & Figure 3g & h).

Table 1: Viability of different cancer cell lines upon treatment with different chemotherapeutics at 48h

	Drugs															
	Doxorubicin (µM)				Taxol (nM)				Bortezomib (nM)				Fludarabine (µM)			
Cell line	0	2	5	10	0	5	10	20	0	5	10	20	0	1	3	5
BT474																
Viability (%)	91.1 89.3 87.0	82.7 77.1 81.0	74.4 72.8 70.2	71.7 67.2 69.3	91.1 89.3 87.0	83.2 87.0 79.6	73.1 76.0 69.7	70.4 73.9 67.8	91.1 89.3 87.0	87.0 81.6 79.7	82.7 79.6 75.3	72.8 68.4 67.9				
Mean	89.1	80.2	72.4	69.4	89.1	83.2	72.9	70.7	89.1	82.7	79.2	69.7				
SD	±2	±2.8	±2.1	±2.2	±2	±3.7	±3.1	±3.2	±2	±3.7	±3.7	±2.6				
SKBR-3																
Viability (%)	85.0 92.0 89.0	77.3 79.3 81.0	71.8 73.3 78.5	70.3 71.9 67.7	85.0 92.0 89.0	81.3 83.2 79.8	79.5 77.4 70.7	72.8 70.8 69.7	85.0 92.0 89.0	81.3 83.2 79.8	79.5 77.4 70.7	72.8 70.8 69.7				
Mean	88.6	79.1	74.5	69.4	88.6	81.4	75.8	71.1	88.6	75.4	68.8	63.7				
SD	±3.5	±1.8	±3.5	±2.1	±3.5	±1.7	±4.5	±1.5	±3.5	±3.9	±1.4	±3.5				
Raji																
Viability (%)									91.0 93.2 89.5	87.6 79.8 83.7	75.2 77.9 71.4	70.7 67.8 59.7	91.0 93.2 89.5	83.4 80.4 79.7	77.8 73.4 69.3	72.9 70.5 66.7
Mean									91.2	83.7	74.8	66.0	91.2	81.2	73.5	70.0
SD									±1.8	±3.9	±3.2	±5.7	±1.8	±1.9	±4.2	±3.1

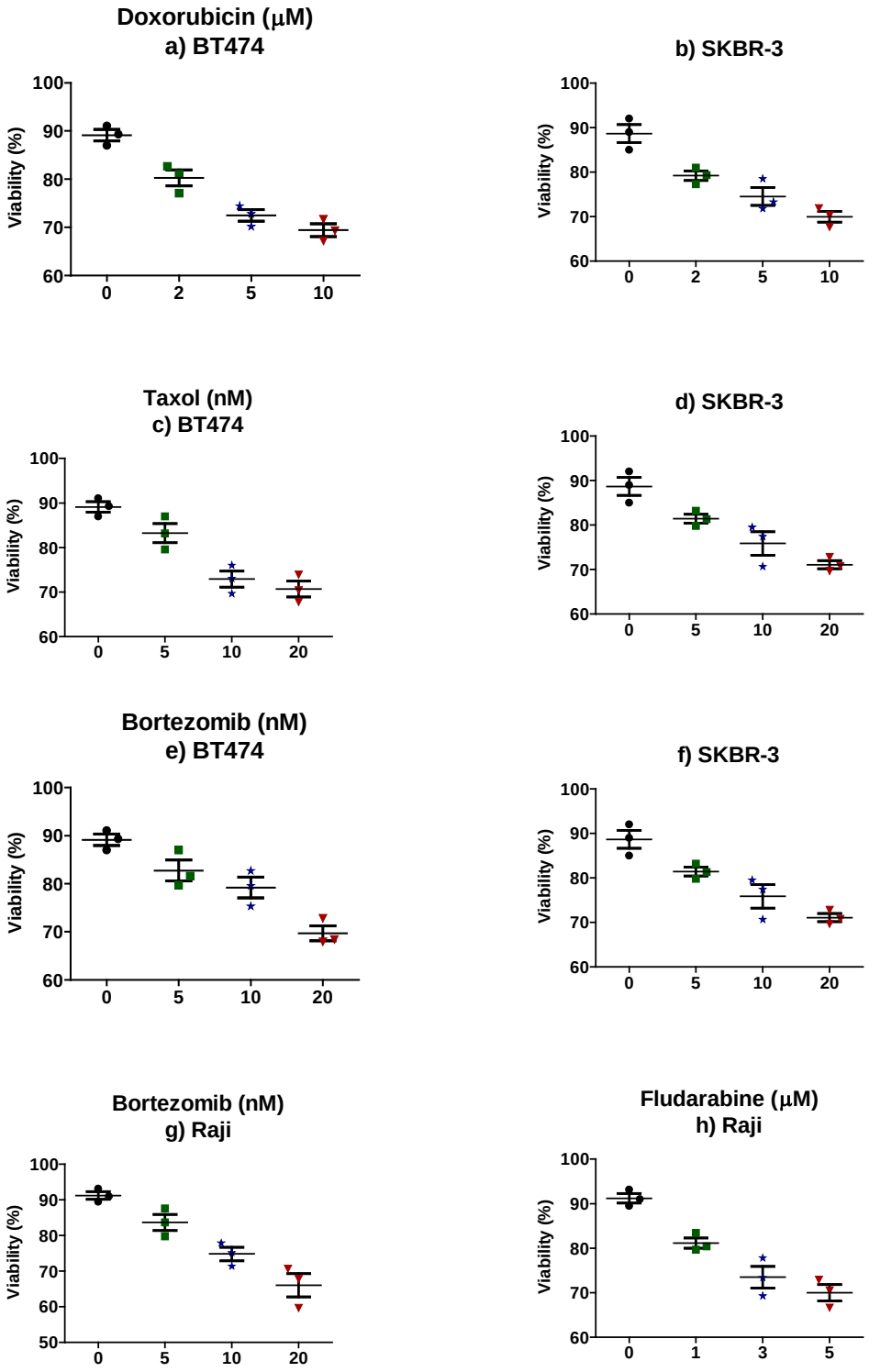


Fig. 3: Viability of different cancer cell lines upon treatment with different chemotherapeutics for 48h. (a&b) Viability of doxorubicin-treated BT474 & SKBR-3 cells. **(c&d**) Viability of taxol-treated BT474 & SKBR-3 cells. **(e, f & g)** Viability of bortezomib-treated BT474, SKBR-3 & Raji cells. **(h)** Viability of fludarabine-treated Raji cells. All cancer cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of each anti-cancer drug. Trypan blue exclusion assay was used to determine cell viability as described in Methods. Each symbol represents an independent experiment. Horizontal line: mean of three experiments; vertical line: SD.

3.2. Impact of doxorubicin on breast cancer cell lines

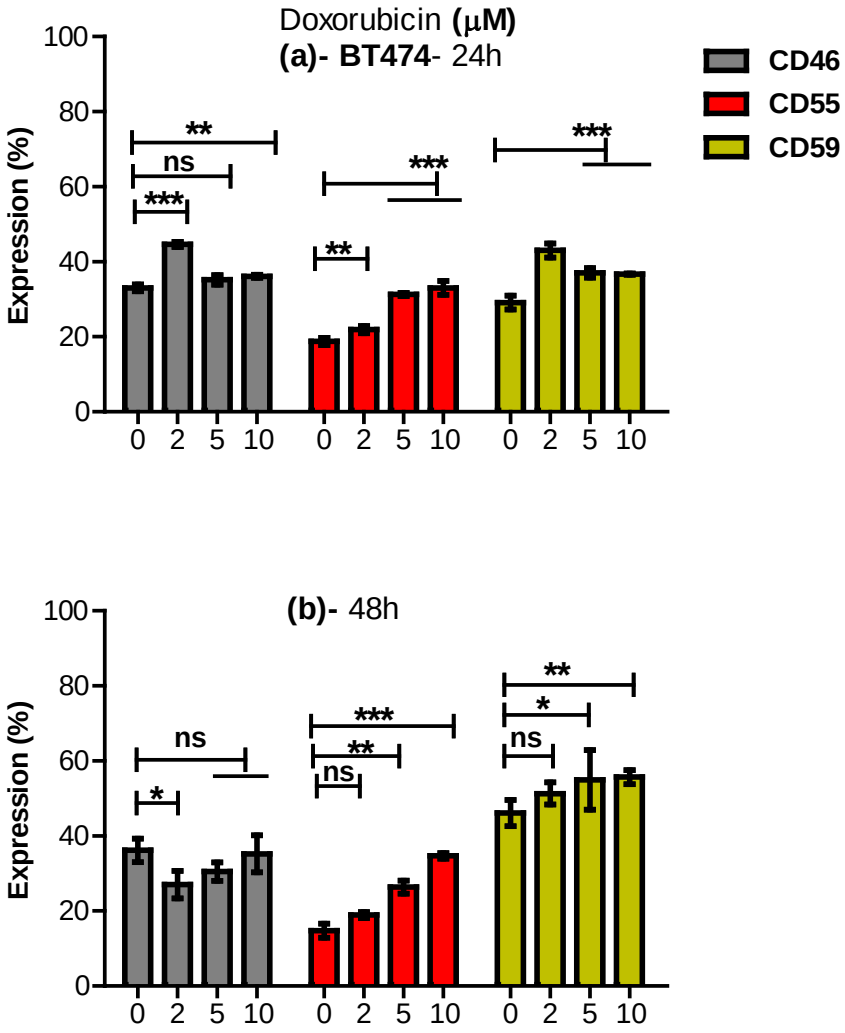
3.2.1. Expression of CD46, CD55 and CD59

33% of untreated BT474 cells were CD46 positive at 24h. Upon treatment with doxorubicin, 44.6%, 35.1% and 36.1% of the given cells were positive for this regulator with an increase by 11.6% at 2μM ($p < 0.001$), 2.1% at 5μM ($p > 0.05$) and 3.1% at 10μM ($p < 0.01$) drug concentration. 18.7% of untreated cells stained positive for CD55. 21.9%, 31.2% and 33% of the drug-treated cells were positive for this regulator with a significant increase by 3.2% ($p < 0.01$), 12.5% and 14.3% ($p < 0.001$) at 2μM, 5μM and 10μM, respectively. 29.1% of untreated cells were CD59 positive. 43%, 37% and 36% of the cells were positive for this regulator with a significant increase by 13.9%, 7.9% and 6.9% ($p < 0.001$) at 2μM, 5μM and 10μM, respectively (Figure 4a).

At 48h, 36.2% of untreated BT474 cells were CD46 positive. Upon treatment with doxorubicin, 27%, 30.5% and 35.2% of the cells were positive for this regulator with a significant reduction by 9.2% ($p < 0.05$), 5.7% and 1% ($p > 0.05$) at 2μM, 5μM and 10μM drug concentration, respectively. 14.7% of untreated cells were CD55 positive. 18.9%, 26.3% and 34.7% of the cells were positive for this regulator with an increase by 4.2% ($p > 0.05$), 11.6% ($p < 0.01$) and 20% ($p < 0.001$) at 2μM, 5μM and 10μM drug concentration, respectively. 46.1% of untreated cells were CD59 positive. Upon treatment with doxorubicin, 51.3%, 54.9% and 55.6% of the cells stained positive for this regulator with an increase by 5.2% ($p > 0.05$), 8.8% ($p < 0.05$) and 9.5% ($p < 0.01$) at 2μM, 5μM and 10μM, respectively (Figure 4b).

41.3% of untreated SKBR-3 cells were CD46 positive at 24h. 59.9%, 62.2% and 72.3% of the cells stained positive for this regulator with a significant increase by 18.6%, 20.9% and 31% ($p < 0.001$ at 2μM, 5μM and 10μM drug concentration), respectively. 25.8% of untreated cells were CD55 positive. 34.5%, 43.9% and 60.6% of the cells were positive for this mCRP with a significant increase by 8.7% ($p < 0.05$), 18.1% and 34.8% ($p < 0.001$) at 2μM, 5μM and 10μM, respectively. 67.1% of untreated cells were CD59 positive. Upon treatment, 73.5%, 74.2% and 79.8% of the cells were positive for this inhibitor with an increase by 6.4% ($p > 0.05$), 7.1% ($p > 0.05$) and 12.7% ($p < 0.001$) at 2μM, 5μM and 10μM drug concentration, respectively (Figure 4c).

At 48h, 46.5% of untreated cells were CD46 positive. 33%, 46.2% and 52.3% of the cells stained positive for this mCRP with a significant reduction by 13.5% at 2µM ($p < 0.001$), without change at 5µM, but with a significant increase by 5.8% at 10µM drug concentration. 57.5% of untreated cells stained positive for CD55. Upon treatment, 38%, 48.8% and 52.5% of the cells were positive for this regulator with a significant reduction by 19.5% ($p < 0.001$), 8.7% ($p < 0.001$) and 5% ($p < 0.01$), at 2µM, 5µM and 10µM drug concentration, respectively. 69% of untreated cells were CD59 positive. Upon treatment, 66%, 70% and 69.8% of the cells were positive for this inhibitor at 2µM, 5µM and 10µM drug concentration, respectively ($p > 0.05$) (Figure 4d).



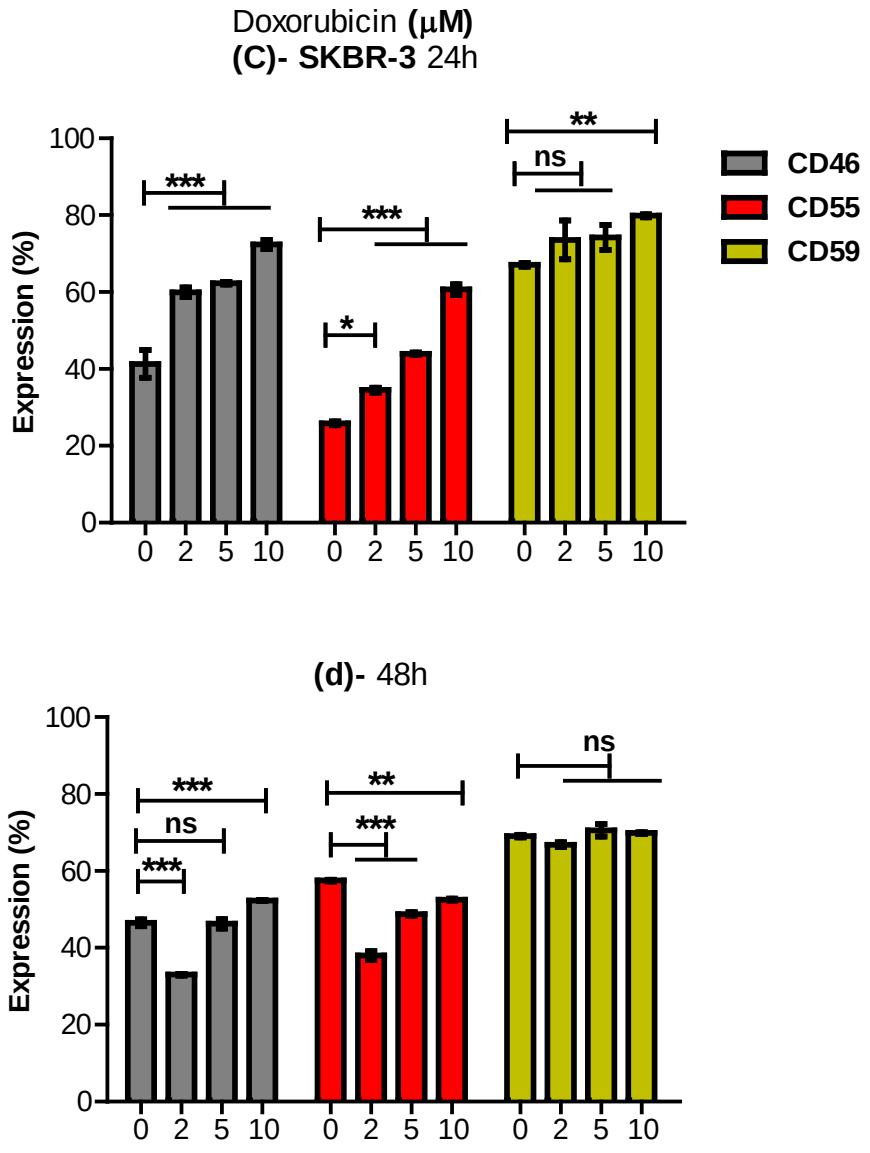


Fig. 4: Expression of CD46, CD55 and CD59 by doxorubicin-treated breast cancer cell lines. (a&b) BT474 cells pre-treated for 24h & 48h. **(c&d)** SKBR-3 cells pre-treated for 24h & 48h. Tumor cells ($5x10^5$/well) were allowed to grow for 24h and 48h either without treatment or with indicated drug concentrations. The monoclonal ms α human CD59, CD55 and CD46 or ms IgG1 isotype control (10µg/ml) were used as a primary antibody, followed by goat α ms IgG-FITC (1:50). The data are presented as mean values ± SD for mCRP positive cells (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences to drug-untreated cells are indicated by asterisks, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) whilst (ns) indicates non-significant differences. Two-way ANOVA, followed by Bonferroni *post hoc* test were used for statistical analysis.

3.2.2. **Complement fH binding**

20.3% of untreated BT474 cells were positive for fH binding at 48h. In doxorubicin-treated cells, 61%, 99.6% and 84.3% of the cells were positive for fH binding with an increase by 40.7% ($p > 0.05$), 79.3% ($p < 0.01$) and 64% ($p < 0.05$) at 2µM, 5µM and 10µM drug concentration, respectively (Figure 5a).

36% of untreated SKBR-3 cells stained positive for fH binding at 48h. 37% of 2µM ($p > 0.05$), 41.4% of 5µM with a 5.4% significant increase ($p < 0.01$) and 32.5% with a 3.5% significant decrease of 10µM doxorubicin-treated cells were positive for fH binding ($p < 0.01$; Figure 5b).

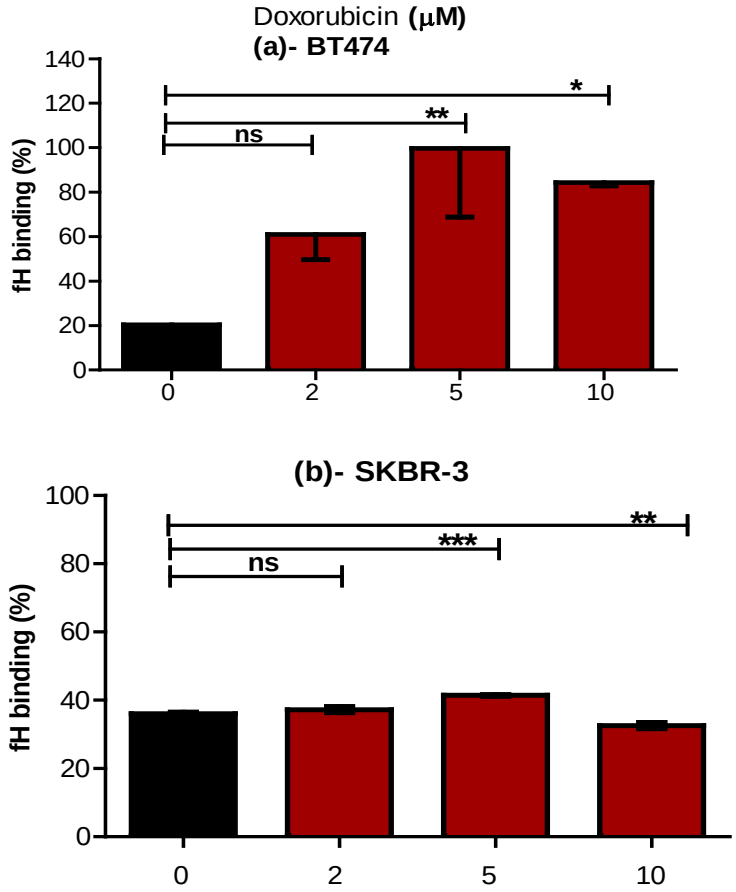


Fig. 5: Complement fH binding to doxorubicin-treated breast cancer cell lines. (a) Complement fH-binding to BT474 cells. **(b)** Complement fH-binding to SKBR-3 cells. Tumor cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of doxorubicin. The cells were incubated with NHS (1:4), washed and stained with polyclonal goat α hum fH primary antibody (1:500), followed by Alexa Fluor 488-Labeled rb α gt IgG (1:800). Goat pre-immune serum was used as a control. The data are presented as mean values ± SD for fH-positive cells (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.001$ (***) whilst (ns) indicates non-significant differences. One-way ANOVA, followed by *Dunn's Multiple Comparison* test was used for statistical analysis.

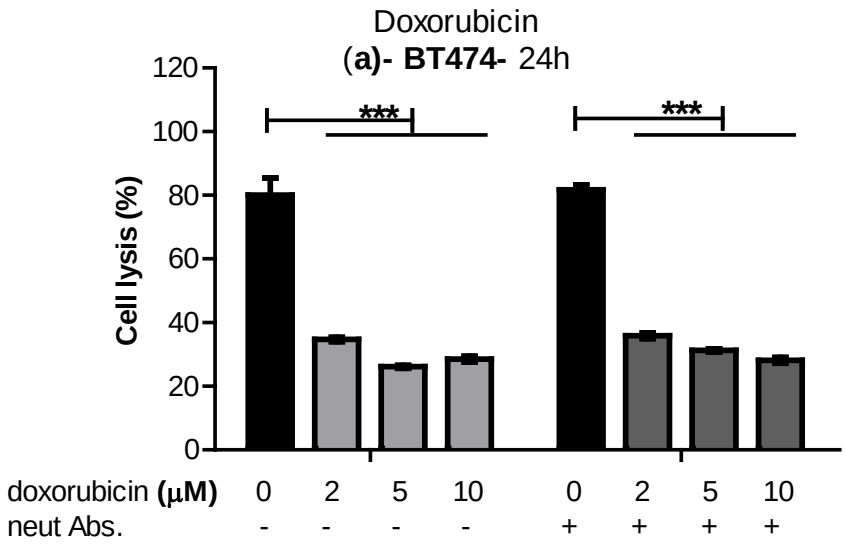
3.2.3. **Cell lysis by CDC**

80% of untreated BT474 cells were lysed by CDC at 24h. 34.7%, 26.1% and 28.5% of doxorubicin-treated cells were lysed at 2μM, 5μM and 10μM with a significant reduction by 45.3%, 53.9% and 51.5%, respectively ($p < 0.001$). In presence of mCRP neutralizing Abs, 81.7% of untreated cells were lysed. 35.7%, 31.2% and 28.1% of treated cells were lysed at 2μM, 5μM and 10μM drug concentration with a significant reduction by 45.9%, 50.5% and 53.6% ($p < 0.001$), respectively (Figure 6a).

At 48h, 92% of untreated cells were lysed. 25.2%, 17.2% and 20.4% of the cells were lysed at 2, 5 and 10μM doxorubicin with a significant reduction by 66.8%, 74.8% and 71.6%, respectively ($p < 0.001$). In presence of mCRP neutralizing Abs, 83% of untreated cells were lysed. 23.4%, 18% and 21.3% of the cells were lysed at 2μM, 5μM and 10μM drug concentration with a significant reduction by 59.6%, 65% and 61.7%, respectively ($p < 0.001$; Figure 6b).

At 24h, 57.1% of untreated SKBR-3 cells were lysed. 20.2%, 28.5% and 35% of the cells were lysed at 2μM, 5μM and 10μM doxorubicin with a significant reduction by 36.9%, 28.6% and 22.1%, respectively ($p < 0.001$). In presence of mCRP neutralizing Abs, 63.4% of untreated cells were lysed. 32.4%, 39.4% and 37.1% of the cells were lysed at 2μM, 5μM and 10μM drug concentration with a significant reduction by 31%, 24% and 26.3% respectively ($p < 0.001$; Figure 6c).

At 48h, 54.8% of untreated cells were lysed. 41.9% were lysed at 2μM, with a significant reduction by 12.9% ($p < 0.01$). 61.7% were lysed at 5μM with an increase by 6.9% and 55.5% were lysed at 10μM drug concentration. In presence of mCRP neutralising Abs 52.2% of untreated cells were lysed. 37.8% were lysed at 2μM, with a significant reduction by 14.4% ($p < 0.01$), 57.1% were lysed at 5μM, with an increase by 4.9% ($P > 0.05$) and 63.3% were lysed at 10μM drug concentration with an increase by 11.1% ($p < 0.01$; Figure 6d).


Doxorubicin
(a)- BT474- 24h
Cell lysis (%)
120
100
80
60
40
20
0
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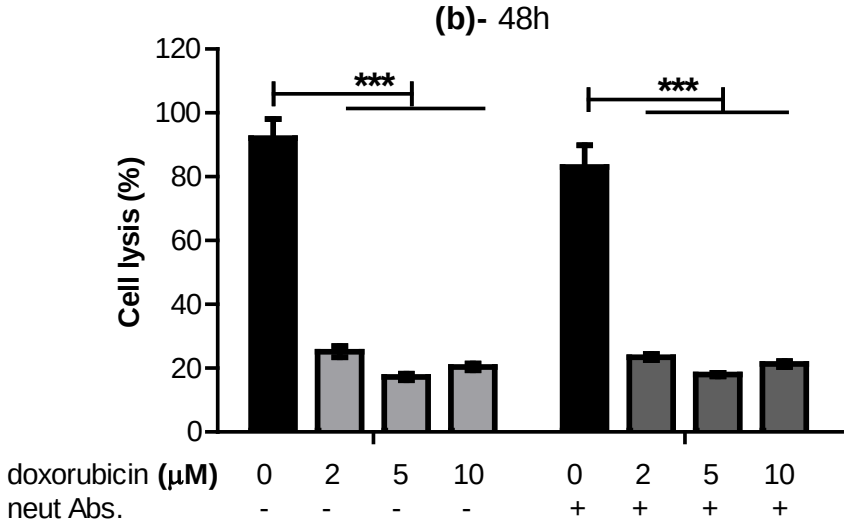
***
doxorubicin (μM) 0 2 5 10 0 2 5 10
neut Abs. - - - - + + + +


(b)- 48h
Cell lysis (%)
120
100
80
60
40
20
0
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doxorubicin (μM) 0 2 5 10 0 2 5 10
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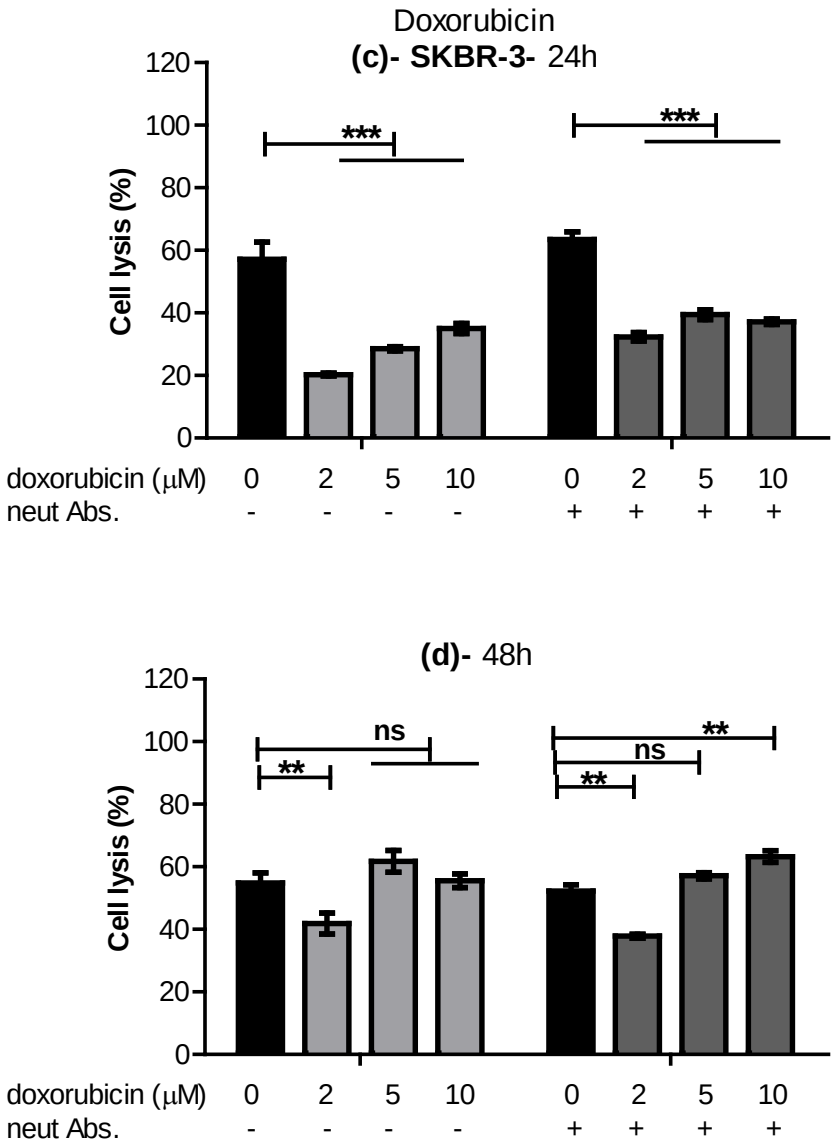


Fig. 6: CDC of doxorubicin-treated breast cancer cell lines. (a&b) CDC of BT474 cells at 24& 48h. **(c&d)** CDC of SKBR-3 cells at 24h & 48h. Tumor cells ($5x10^5$/well) were allowed to grow for 24h and 48h either without treatment or with indicated concentrations of doxorubicin. The cells were labelled with ^{51}Cr and then incubated with α tumor antibody (1:50) in absence or presence of mCRP specific neutralizing Abs (2μg/ml). Human serum (25%) was used as a complement source. The data are presented as mean values ± SD for CDC (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.01$ (**), $p < 0.001$ (***) whilst (ns) indicates non-significant differences. Two-way ANOVA, followed by Bonferroni *post hoc* test was used for statistical analysis.

3.2.4. **Opsonisation with iC3b**

50.5% of untreated BT474 cells were opsonised with iC3b at 48h. 64.6% of 2µM doxorubicin-treated cells stained positive for iC3b with a significant increase by 14.1% ($p < 0.05$), 55.1% of 5µM drug-treated cells were positive for iC3b with an increase by 4.6% ($p > 0.05$) and 60.5% of 10µM treated cells were opssonised with iC3b with an increase by 10% ($p > 0.05$; Figure 7a).

43.3% of untreated SKBR-3 cells were opsonised with iC3b. 48.7% of 2µM doxorubicin-treated cells stained positive for iC3b with an increase by 5.4% ($p > 0.05$), 60.9% of the cells treated with either 5µM or 10µM doxorubicin stained positive for iC3b with a significant increase by 17.6% with the both concentrations ($p < 0.01$; Figure 7b).

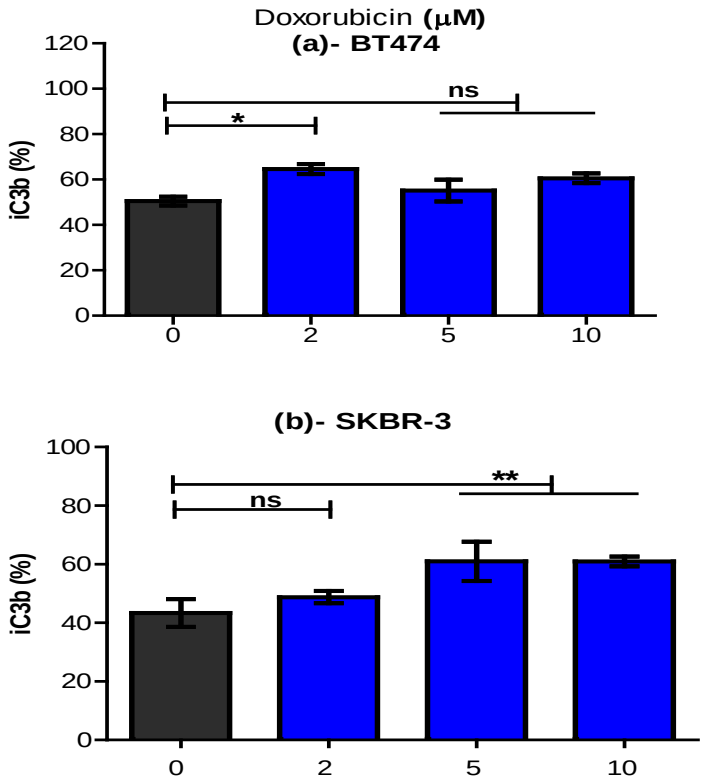


Fig. 7: Opsonisation of doxorubicin-treated breast cancer cell lines with iC3b. (a) BT474 cells. **(b)** SKBR-3 cells. Tumor cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of doxorubicin. The cells were incubated with α tumor antibodies (1:50), followed by adding either C8-depleted or heat-inactivated serum as a complement source. The cells were stained with monoclonal ms α human iC3b primary antibody (10µg/ml), followed by goat α ms IgG-FITC (1:50). The data are presented as mean values ± SD for iC3b positive cells (in %) are presented. In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.01$ (**) whilst (ns) indicates non-significant differences. One-way ANOVA, followed by *Dunn's Multiple Comparison* test was used for statistical analysis.

3.3. Impact of taxol on breast cancer cell lines

3.3.1. Expression of CD46, CD55 and CD59

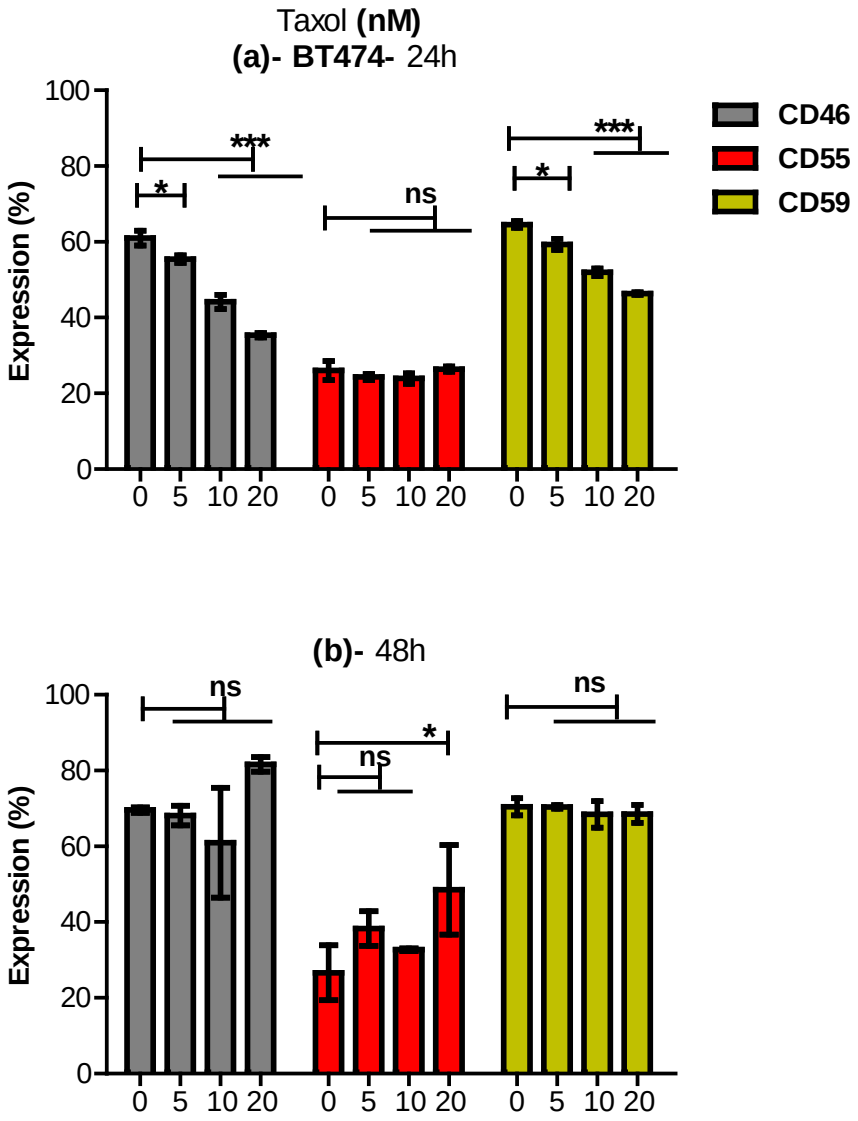
61% of untreated BT474 cells were CD46 positive at 24h. Upon treatment, 55.4%, 44.1% and 35.4% of the cells were positive for this regulator at 5nM, 10nM and 20nM taxol with a significant reduction by 5.6% ($p < 0.05$), 16.9% and 25.6% ($p < 0.001$), respectively. 26% of untreated cells were CD55 positive. 24%, 23.9% and 26.4% of the cells stained positive for this regulator at 5nM, 10nM and 20nM drug concentration, respectively. 64.5% of untreated cells were CD59 positive. Upon treatment, 59.3% of 5nM taxol-treated cells were positive with a significant reduction by 5.2% ($p < 0.05$), 51.9% of 10nM taxol-treated cells stained positive for this regulator with a significant reduction by 12.6% ($p < 0.001$) and 46.3% of 20nM taxol-treated cells were positive for this mCRP with a significant reduction by 18.2% ($p < 0.001$; Figure 8a).

At 48h, 69.6% of untreated cells stained positive for CD46. Upon treatment, 68.1%, 60.9% and 81.6% of the cells were positive for this regulator at 5nM, 10nM and 20nM taxol (each n.s.), respectively. 26.6% of the given untreated cells were CD55 positive. 38.2% and 32.7% of the cells were positive for this regulator at 5nM and 10nM taxol, respectively ($p > 0.05$) whereas 48.5% of 20nm taxol-treated cells were positive for the CD46 with a significant increase by 21.9% ($p < 0.05$). 70.4% of untreated cells were CD59 positive. Upon treatment, 70.4%, 68.4% and 68.5% of the cells were CD46 positive at 5nM, 10nM and 20nM taxol, respectively (Figure 8b).

17% of untreated SKBR-3 cells were CD46 positive at 24h. upon treatment, 44.3%, 47.6% and 62.8% of the cells were positive for this inhibitor with a significant increase by 27.3%, 30.6% and 45.8% at 5nM, 10nM and 20nM taxol, respectively ($p < 0.001$). 17.6% of untreated cells were positive for CD55. 29.8%, 31.2% and 40.3% of the cells stained positive for the CD55 with a significant increase by 12.2% ($p < 0.01$), 13.6% and 22.7% ($p < 0.001$) at 5nM, 10nM and 20nM drug concentration, respectively. 39.6% of untreated cells were CD59 positive. After treatment, 58.2%, 58.3% and 68.7% of the cells were positive for this regulator with a significant increase by 18.6%, 18.7% and 29.1% at 5nM, 10nM and 20nM taxol, respectively ($p < 0.001$; Figure 8c).

At 48h, 18% of untreated SKBR-3 cells were CD46 positive. After treatment, 42.2%, 41.6% and 48% of the cells were positive for this regulator

with a significant increase by 24.2%, 23.6% and 30% at 5nM, 10nM and 20nM taxol, respectively ($p < 0.001$). 34.1% of untreated cells stained positive for CD55. Upon treatment, 31.4% of 5nM taxol-treated cells were positive for this regulator with a 2.7% reduction ($p > 0.05$), 25.7% of 10nM taxol-treated cells were positive for this regulator with 8.7 reduction ($p > 0.05$) and 26.2% of 20nM taxol-treated cells were positive for this regulator with 7.9% reduction ($p < 0.05$). 51.3% of untreated cells were CD59 positive. Upon treatment, 61.4% of 10nM taxol-treated cells were positive for this inhibitor ($p > 0.05$), 50.5% and 62.6% of 5nM and 20nM taxol-treated cells were positive for this regulator with a significant increase by 10.1% and 11.3%, respectively ($p < 0.01$; Figure 8d).


Taxol (nM)
(a)- BT474- 24h
Expression (%)
100
80
60
40
20
0
***
*
ns
***
*
CD46
CD55
CD59
0 5 10 20 0 5 10 20 0 5 10 20
(b)- 48h
Expression (%)
ns
ns
*
ns
0 5 10 20 0 5 10 20 0 5 10 20

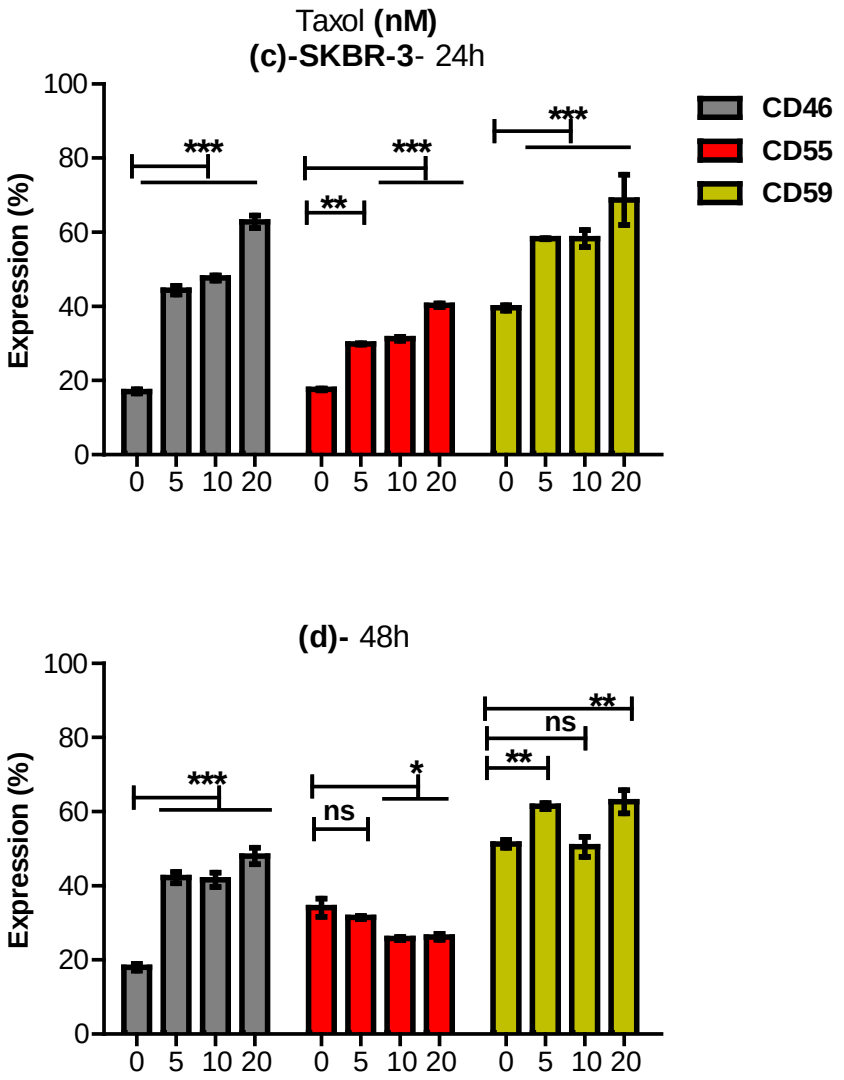


Fig. 8: Expression of CD46, CD55 and CD59 by taxol-treated breast cancer cell lines. (a&b) BT474 cells pre-treated for 24h & 48h. **(c&d)** SKBR-3 cells pre-treated for 24h & 48h. Tumor cells ($5x10^5$/well) were allowed to grow for 24 and 48h either without treatment or with indicated drug concentrations. The cells were stained with ms IgG1 isotype control or with monoclonal ms α human CD59, CD55 and CD46 primary antibodies (10µg/ml), followed by goat α ms IgG-FITC (1:50). The data are presented as mean values ± SD for cells staining positive for mCRP (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) whilst (ns) indicates non-significant differences. Two-way ANOVA, followed by Bonferroni *post hoc* test was used for statistical analysis.

3.3.2. **Complement fH binding**

25.1% of untreated BT474 cells were positive for fH binding at 48h. 23.7% of 5nM taxol-treated cells stained positive for fH. 27.7% of 10nM taxol-trreated cells were positive for fH bindinig with a significant increase by 2.6% (p

< 0.01). 33% of 20nM drug-treated cells stained positive for fH bindinig with an increase by 7.3% ($p < 0.001$; Figure 9a).

32% of untreated SKBR-3 cells stained positive for fH at 48h. 34% and 32% of 5nM and 10nM taxol-treated cells were positive for fH, wereas, 39% of 20nM taxol-treated cells stained positive for fH with a 7% significant increase ($p < 0.05$; Figure 9b).

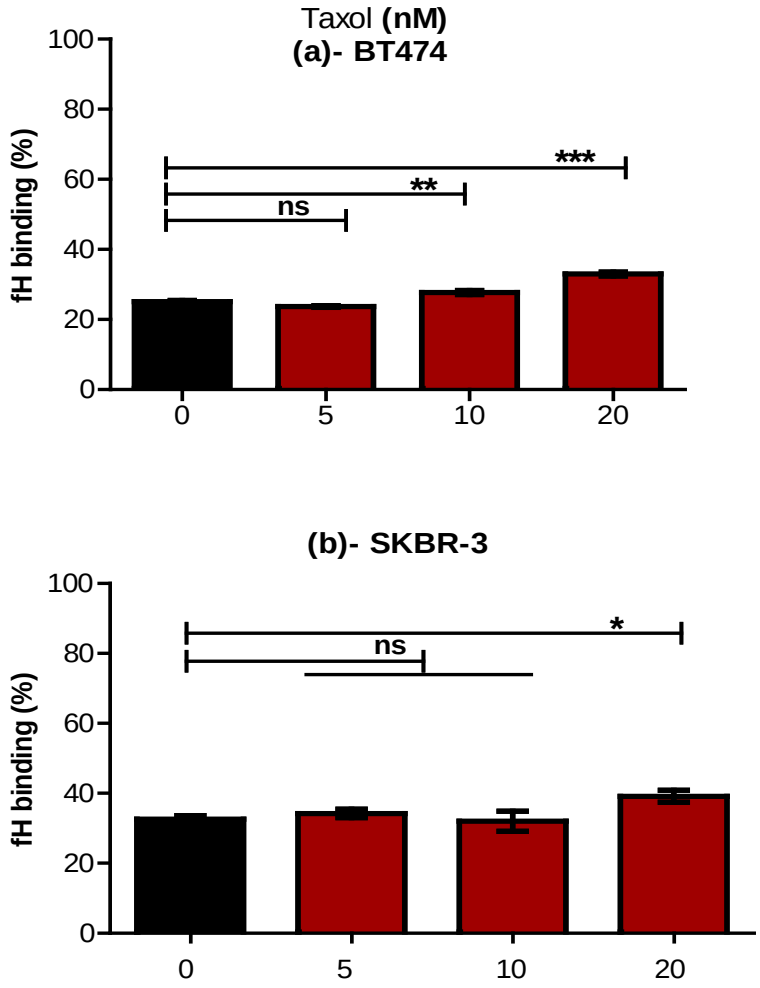


Fig. 9: Complement fH binding to taxol-treated breast cancer cell lines. (a) fH-binding to BT474 cells. **(b)** fH-binding to SKBR-3 cells. Tumor cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of taxol. These cells were incubated with NHS (1:4), washed and stained with polyclonal goat α human fH primary antibody (1:500), followed by Alexa Fluor 488-Labeled rb α gt IgG (1:800). Goat pre-immune serum was used as a control. The data are presented as mean values ± SD for fH positive cells (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.001$ (***) whilst (ns) indicates non-significant differences. One-way ANOVA, followed by *Dunn's Multiple Comparison* test was used for statistical analysis.

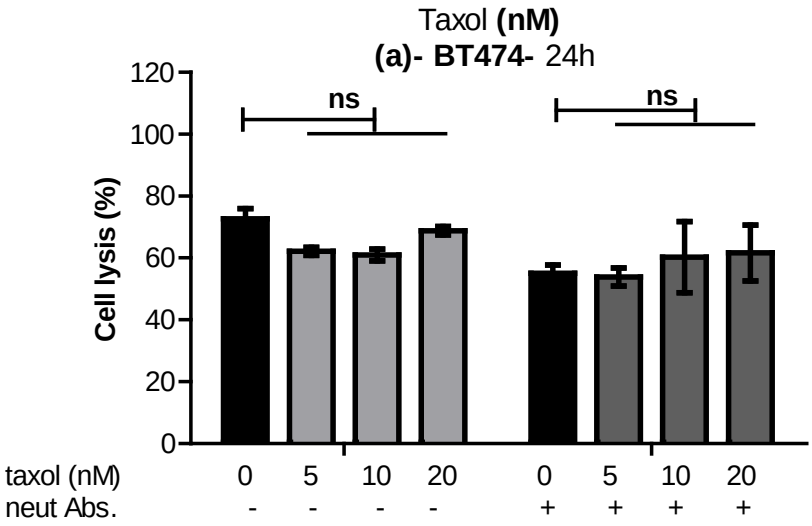
3.3.3. **Cell lysis by CDC**

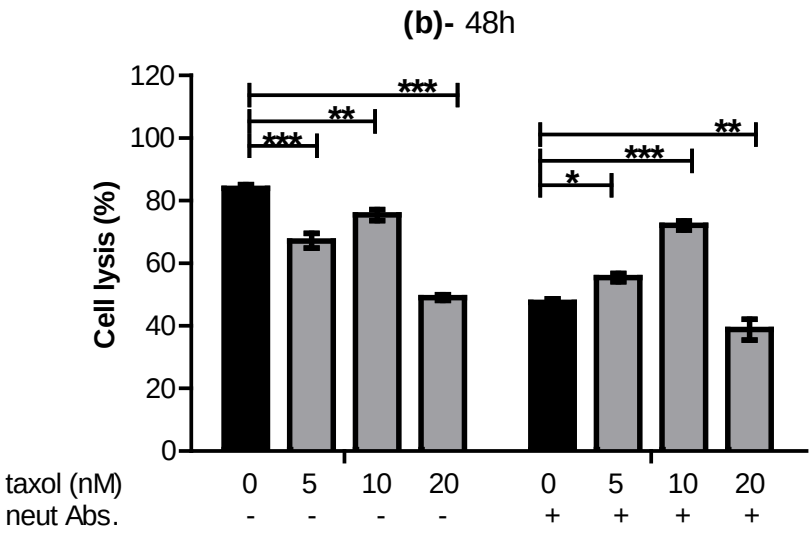
72.6% of untreated BT474 cells were lysed at 24h by CDC. 62.3%, 60.9% and 68.7% of the cells were lysed with a reduction by 10.3%, 11.7% and 3.9% at 5nM, 10nM and 20nM taxol ($p > 0.05$), respectively. In presence of mCRP neutralizing Abs, 55% of untreated cells were lysed by CDC. 53.8% of 5nM, 60.2% of 10nM with an increase by 5.2% and 61.6% of 20nM taxol-treated cells were lysed with an increase by 6.6% (Figure 10a).

At 48h, 83.9% of untreated BT474 cells were lysed. 67.11% of 5nM taxol-treated cells were lysed with a significant reduction by 16.1% ($p < 0.001$), 75.41% of 10nM taxol-treated cells were lysed with a significant reduction by 8.4% ($p < 0.01$) and 49% of 20nM taxol-treated cells were lysed with a significant reduction by 34.9% ($p < 0.001$). In presence of mCRP neutralising Abs, 47.5% of untreated cells were lysed. 55.4 % of 5nM taxol-treated cells were lysed with a significant increase by 7.9% ($p < 0.05$) , 72.1% of 10nM taxol-treated cells were lysed with a significant increase by 24.6% ($p < 0.001$) and 38.8% of 20nM taxol-treated cells were lysed with a significant decrease by 8.7% ($p < 0.01$; Figure 10b).

81.6% of untreated SKBR-3 cells were lysed at 24h by CDC. 77.7% of 5nM taxol-treated cells were lysed, 65.2% and 39.3% of 10nM and 20nM taxol-treated cells were lysed with a significant reduction by 16.4% ($p <0.01$) and 42.5% ($p < 0.001$), respectively. In presence of mCRP neutralising Abs, 59.9% of untreated cells were lysed. 72.6% of 5nM taxol-treated cells were lysed with a significant increase by 12.7% ($p < 0.01$), 51.3% of 10nM taxol-treated cells were lysed with a reduction by 8.6% ($p > 0.05$) and 41.3% of 20nM taxol-treated cells were lysed with a reduction by 18.6% ($p < 0.001$; Figure 10c).

At 48h, 69.2% of untreated SKBR-3 cells were lysed. 49.5%, 47.8% and 3.5% of 5nM, 10nM and 20nM taxol-treated cells were lysed with a significant decrease by 19.7%, 21.4% and 65.7%, respectively ($p < 0.001$). In presence of mCRP neutralizing Abs, 60.3% of untreated cells were lysed. 67% of 5nM taxol-treated cells were lysed with a significant increase by 6.7% ($p < 0.05$), 53.9% and 5.2% of 10nM and 20nM taxol-treated cells were lysed with a significant decrease by 6.4% and 55.1%, respectively ($p < 0.001$; Figure 10d).


Taxol (nM)
(a)- BT474- 24h
Cell lysis (%)
120
100
80
60
40
20
0
ns
ns
taxol (nM)
0 5 10 20 0 5 10 20
neut Abs.
- - - - + + + +


(b)- 48h
Cell lysis (%)
120
100
80
60
40
20
0
***
**
***
**
***
*
taxol (nM)
0 5 10 20 0 5 10 20
neut Abs.
- - - - + + + +

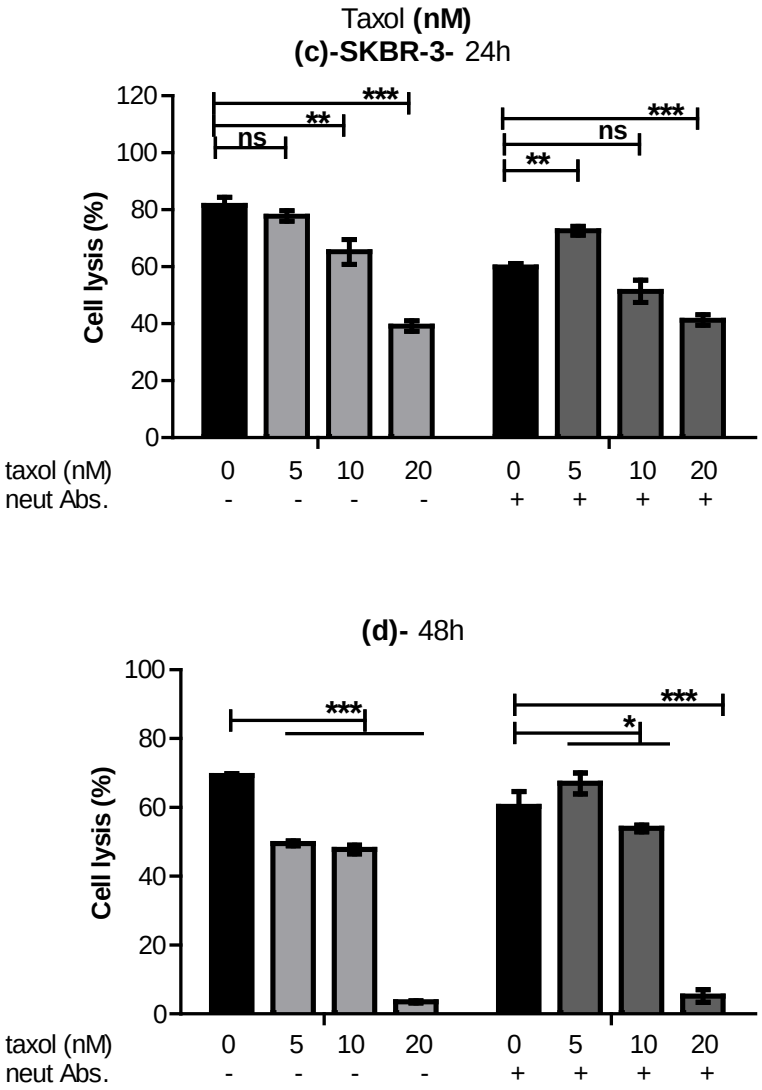


Fig. 10: CDC of taxol-treated breast cancer cell lines.
(a&b) CDC of BT474 cells at 24& 48h. **(c&d)** CDC of SKBR-3 cells at 24h & 48h. Tumor cells ($5x10^5$/well) were allowed to grow for 24h and 48h either without treatment or with indicated concentrations of taxol. The cells were labelled with ^{51}Cr and then incubated with α tumor antibodies (1:50) in absence or presence of mCRP neutralising antibodies (2µg/ml). Human serum (25%) was used as a complement source. The data are presented as mean values ± SD for CDC (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.01$ (**), $p < 0.001$ (***) whilst (ns) indicates non-significant differences). Two-way ANOVA, followed by Bonferroni *post hoc* test was used for statistical analysis.

3.3.4. **Opsonisation with iC3b**

50.5% of the untreated BT474 cells were opsonised with iC3b at 48h. 81.4%, 80.1% and 76.3% of the cells stained positive for iC3b with a significant increase by 30.9%, 29.6% and 25.8% at 5nM, 10nM and 20nM taxol, respectively ($p < 0.001$; Figure 11a). 20.7% of untreated SKBR-3 cells were

opsonised with iC3b at 48h. 21.8% of 5nM taxol-treated cells were iC3b positive with an increase by 1.1% ($p > 0.05$), 28.7% of 10nM taxol-treated cells stained positive for iC3b with a significant increase by 8% ($p < 0.01$) and 36.8% of 20nM taxol-treated cells were opsonised with iC3b recording a significant increase by 16.1% ($p < 0.001$; Figure 11b).

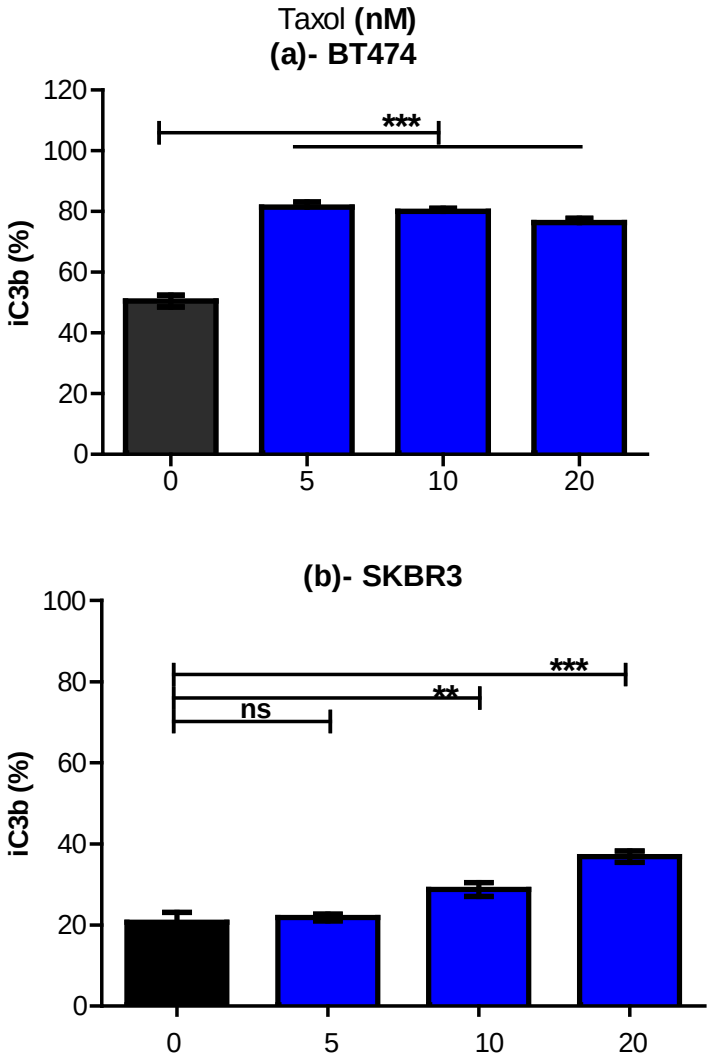


Fig. 11: Opsonisation of taxol-treated breast cancer cell lines with iC3b.
(a) BT474 cells. **(b)** SKBR-3 cells. Tumor cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of taxol. The cells were incubated with anti-tumor antibodies (1:50), followed either by C8-depleted or heat-inactivated serum as a complement source. The cells were stained with monoclonal ms α human iC3b primary antibody (10µg/ml), followed by goat α ms IgG-FITC (1:50). The data are presented as mean values ± SD for iC3b positive cells (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.01$ (**) whilst (ns) indicates non-significant differences. One-way ANOVA, followed by *Dunn's Multiple Comparison* test was used for statistical analysis.

3.4. Impact of bortezomib on different cancer cell lines

3.4.1. Expression of CD46, CD55 and CD59

22.4% of the untreated BT474 cells stained positive for CD46 at 24h. 21.5%, 22.8% and 21.8% of the cells were positive for this regulator at 5nM, 10nM and 20nM bortezomib, respectively ($p > 0.05$). 16.6% of untreated cells were CD55 positive. 16%, 16.3% and 16.2% of 5nM, 10nM and 20nM bortezomib-treated cells were positive for this regulator, respectively ($p > 0.05$). 35% of untreated cells stained positive for CD59. 35.5%, 36% and 36.4% of 5nM, 10nM and 20nM bortezomib-treated cells were positive for this regulator, respectively ($p > 0.05$; Figure 12a).

At 48h, 54.4% of untreated BT474 cells were CD46 positive. 36.8%, 36.7% and 31.4% of 5nM, 10nM and 20nM bortezomib-treated cells stained positive for this regulator with a significant reduction by 17.6%, 17.7% and 23%, respectively ($p < 0.001$). 27.5% of untreated cells stained positive for CD55. 22.9%, 21.9% and 20.9% of 5nM, 10nM and 20nM bortezomib-treated cells were positive for this regulator with a partial decrease by 4.6%, 5.6% and 6.6%, respectively ($p > 0.05$). 63.4% of untreated cells were positive for CD59. 45.2%, 36.8% and 38.3% of 5nM, 10nM and 20nM bortezomib-treated cells stained positive for this regulator with a significant reduction by 18.2%, 26.6% and 25.1%, respectively ($p < 0.001$; Figure 12b).

26.4% of untreated SKBR-3 cells were CD46 positive at 24h. 24.4% and 26.2% of 5nM and 10nM bortezomib-treated cells stained positive for this regulator, respectively ($p > 0.05$), whereas 34.4% of 20nM bortezomib-treated cells were CD46 positive with a significant increase by 8% ($p < 0.001$). 15.1% of untreated cells were CD55 positive. 15.8% and 16.7% of 5nM and 10nM bortezomib-treated cells were positive for this regulator, respectively ($p > 0.05$), whereas 18.1% of 20nM bortezomib-treated cells stained positive for this regulator with a significant increase by 3% ($p < 0.001$). 41% of untreated cells were CD59 positive. 41.4% of 5nM bortezomib-treated cells were positive for this regulator ($p > 0.05$), 44.1% and 50.1% of 10nM and 20nM bortezomib-treated cells stained positive for this regulator with a significant increase by 3.1%; $p < 0.05$ and 9.1%; $p < 0.001$, respectively (Figure 12c).

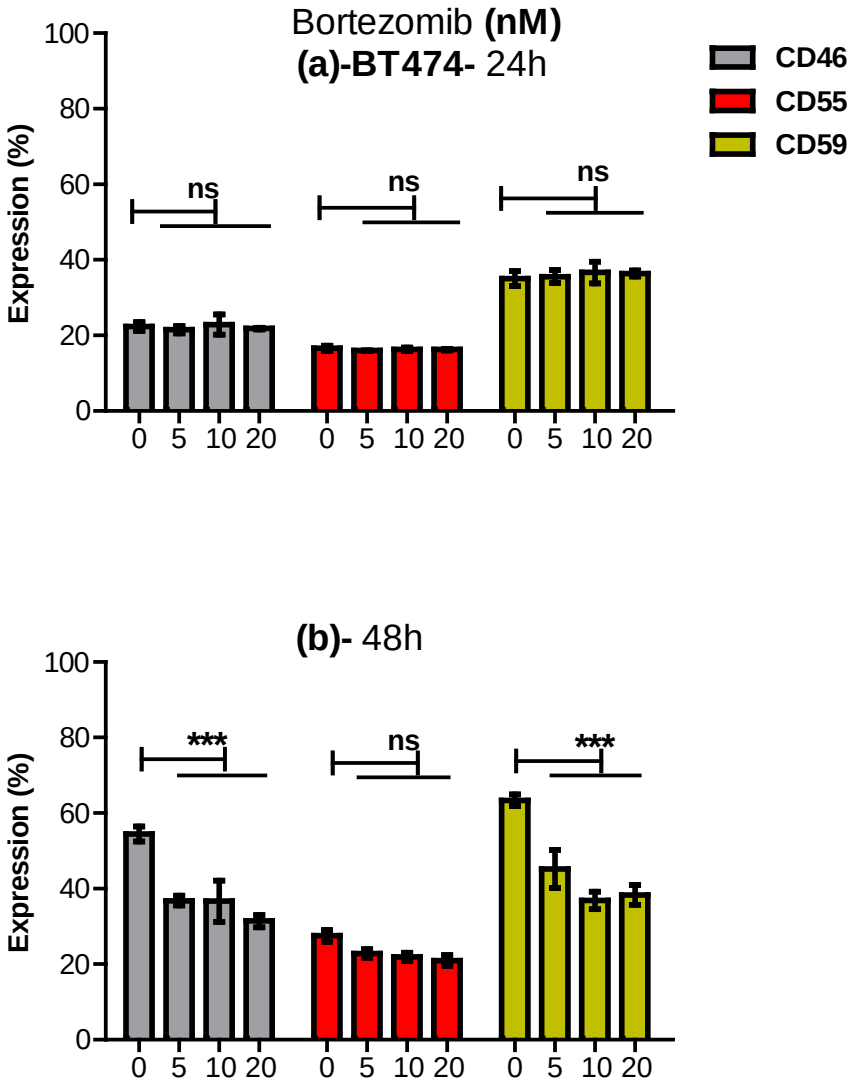
At 48h, 22.8% of untreated cells stained positive for CD46. 22%, 21.1% and 21.2% of 5nM, 10nM and 20nM bortezomib-treated cells were positive for

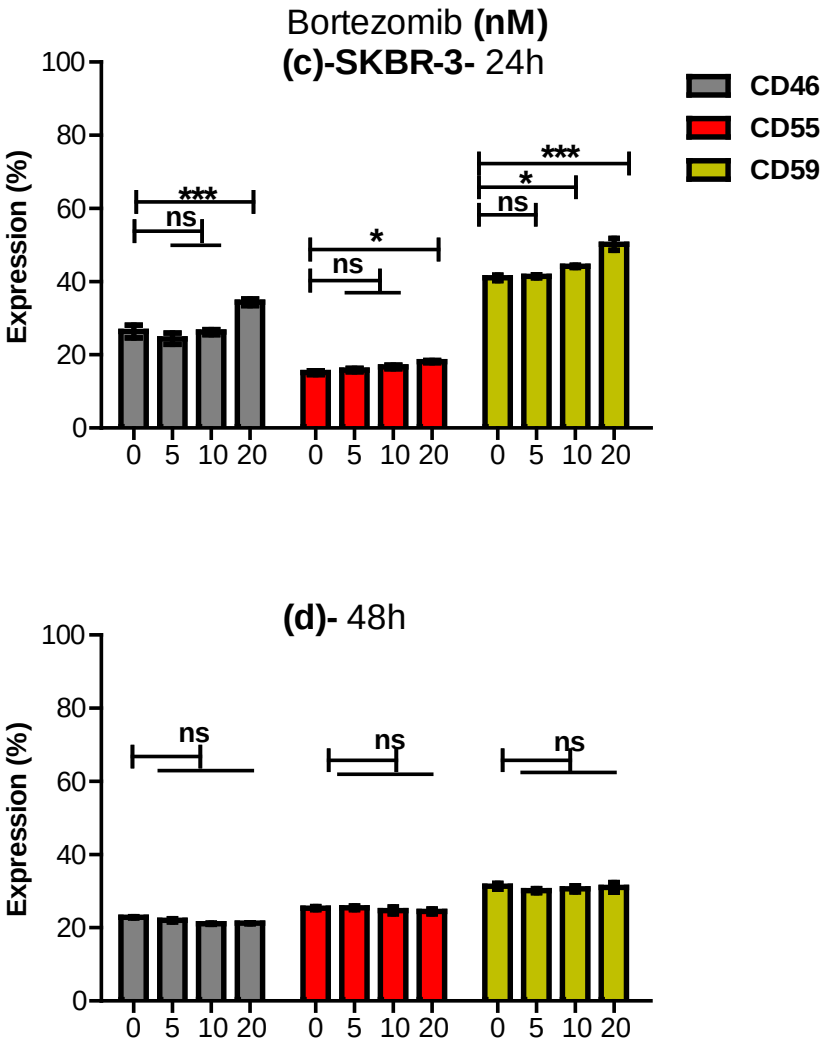
this regulator, respectively ($p > 0.05$). 25.4% of untreated cells were CD55 positive. 25.4%, 24.7% and 24.4% of 5nM, 10nM and 20nM bortezomib-treated cells stained positive for this regulator, respectively ($p > 0.05$). 31.3% of untreated cells stained positive for CD59. 30.1%, 30.6% and 31% of 5nM, 10nM and 20nM bortezomib-treated cells were positive for this regulator, respectively ($p > 0.05$; Figure 12d).

32.9% of untreated Raji cells were CD46 positive at 24h. 34.6% of 5nM bortezomib-treated cells stained positive for this regulator with an increase by 1.7% ($p > 0.05$), 38.3% of 10nM bortezomib-treated cells were CD46 positive with an increase by 5.4% ($p < 0.01$) and 38.9% of 20nM bortezomib-treated cells stained positive for this regulator with an increase by 6% ($p < 0.001$). 30.6% of untreated cells were CD55 positive. 34% of 5nM bortezomib-treated cells were positive for this regulator with a significant increase by 4.6% ($p < 0.05$), 35.5% of 10nM bortezomib-treated cells stained positive for this regulator with a significant increase by 4.9% and 35.8% of 20nM bortezomib-treated cells were CD55 positive with a significant increase by 5.2% ($p < 0.01$). 30.3% of untreated cells were CD59 positive. 32.2%, 35.1% and 34.6% of the cells were positive for this regulator with an increase by 1.9% ($p > 0.05$), 4.8% ($p < 0.001$) and 4.3% ($p < 0.01$) at 5nM, 10nM and 20nM bortezomib, respectively (Figure 12e).

At 48h, 45.5% of untreated cells were CD46 positive. 48.3%, 46.9% and 47.7% of 5nM, 10nM and 20nM bortezomib-treated cells stained positive for this regulator, respectively ($p > 0.05$).

41.2% of unntreated cells were CD55 positive. 40.2%, 41.4% and 41.9% of 5nM, 10nM and 20nM bortezomib-treated cells stained positive for this regulator, respectively ($p > 0.05$). 37.8% of untreated cells were CD59 positive positive. 40.3%, 38% and 38.1% of 5nM, 10nM and 20nM bortezomib-treated cells were positive for this inhibitor, respctively ($p > 0.05$; Figure 12f).


Bortezomib (nM)
(a)-BT474- 24h
CD46
CD55
CD59
Expression (%)
100
80
60
40
20
0
ns
ns
ns
0 5 10 20 0 5 10 20 0 5 10 20
(b)- 48h
***
ns
***


Bortezomib (nM)
(c)-SKBR-3- 24h
Expression (%)
100
80
60
40
20
0
CD46
CD55
CD59
***
ns
*
ns
***
*
ns
0 5 10 20 0 5 10 20 0 5 10 20
(d)- 48h
Expression (%)
ns
ns
ns
0 5 10 20 0 5 10 20 0 5 10 20

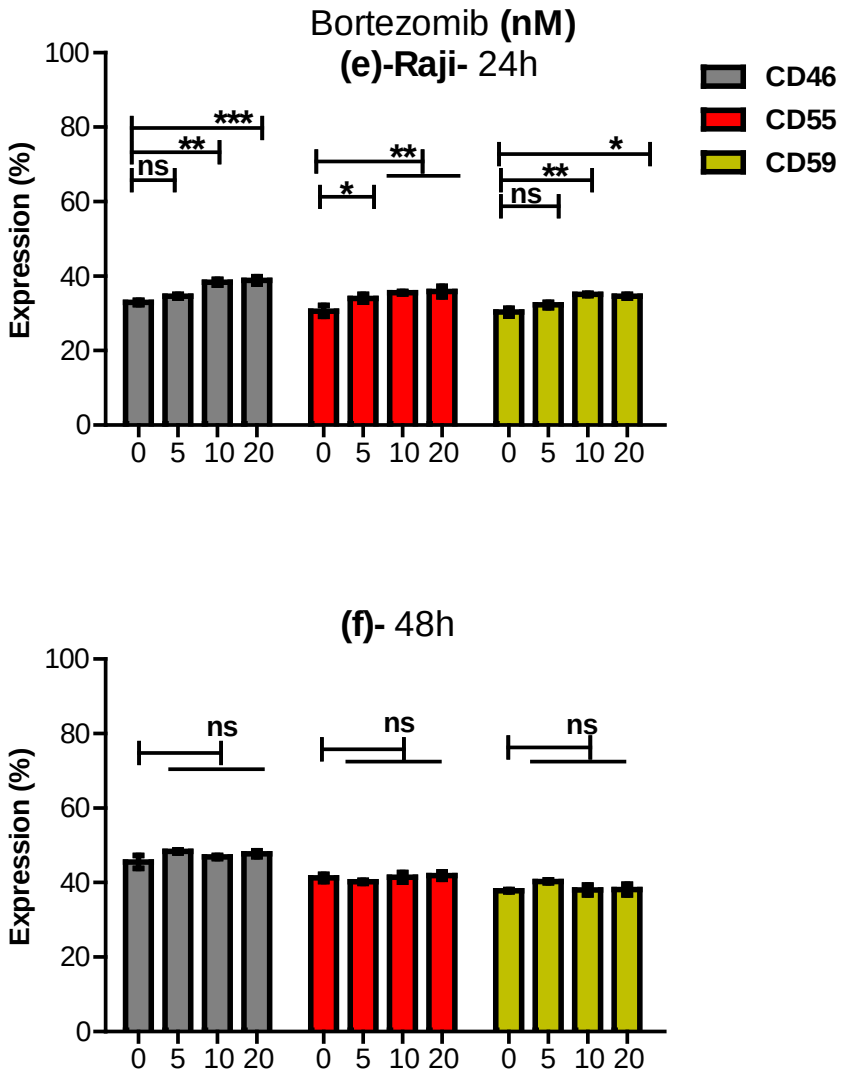


Fig. 12: Expression of CD46, CD55 and CD59 by different bortezomib-treated cancer cell lines. (a&b) BT474 cells at 24 & 48h. **(c&d)** SKBR-3 cells at 24h & 48h. **(e&f)** Raji cells at 24h & 48h.Tumor cells ($5x10^5$/well) were allowed to grow for 24h and 48h either without treatment or with indicated drug concentrations. The cells were stained with ms IgG1 isotype control or with monoclonal ms α hum CD59, CD55 and CD46 primary antibodies (10µg/ml), followed by goat α ms IgG-FITC (1:50). The data are presented as mean values ± SD of mCRP positive tumor cells cells (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) whilst (ns) indicates non-significant differences. Two-way ANOVA, followed by Bonferroni *post hoc* test was used for statistical analysis.

3.4.2. Complement fH binding

20.3% of untreated BT474 cells were positive for fH binding at 48h. 31.3% of the cells stained positive for fH with an increase by 11% at 5nM bortezomib ($p < 0.05$) whereas 67.3% and 51.3% of the cells were positive for fH at 10nM and 20nM bortezomib with a significant increase by 47% ($p < 0.01$) and 31% ($p < 0.05$), respectively (Figure 13a).

19.3% of untreated SKBR-3 cells stained positive for fH binding at 48h. 20.9%, 20.2% and 18.1% of the cells stained positive for fH at 5nM, 10nM and 20nM bortezomib, respectively ($p > 0.05$; Figure 13b).

13.2% of untreated Raji cells were fH positive at 48h. 8.8% of 5nM bortezomib-treated cells stained positive for fH with a significant reduction by 4.4% ($p < 0.05$), 10.9% of 10nM bortezomib-treated cells were fH positive with a reduction by 2.3% ($p > 0.05$) and 23% of 20nM bortezomib-treated cells stained positive for fH with a significant increase by 9.8% ($p < 0.001$; Figure 13c).

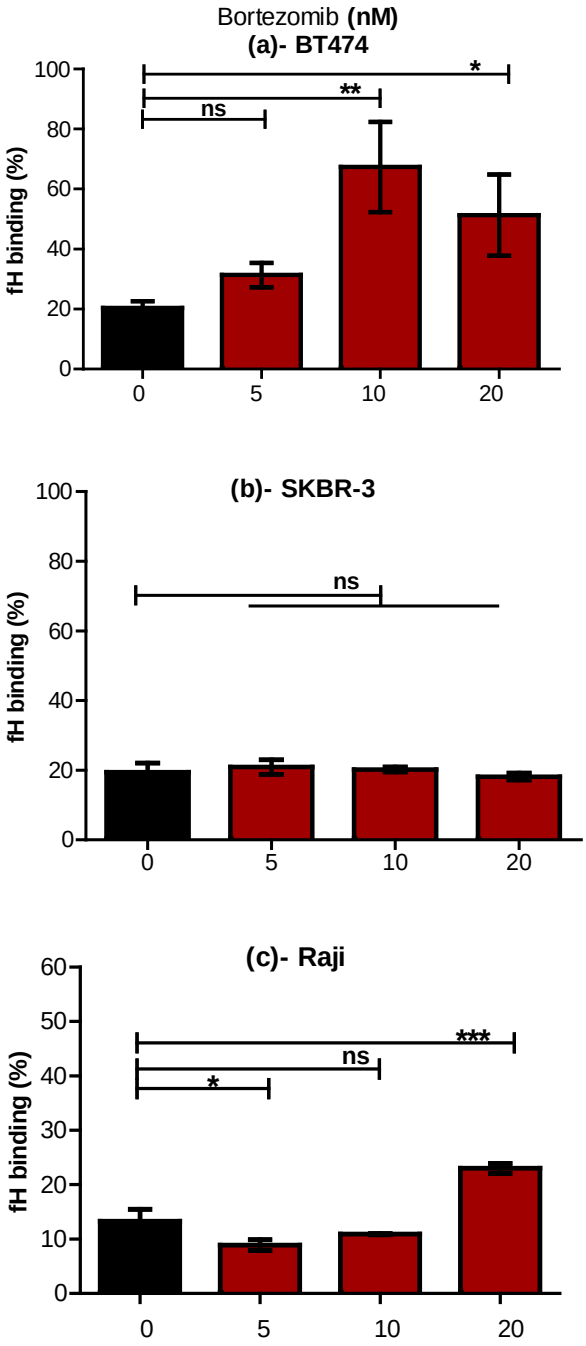


Fig. 13: Complement fH binding to different bortezomib-treated cancer cell lines. (a) fH binding to BT474 cells. **(b)** fH binding to SKBR-3 cells. **(c)** fH binding to Raji cells. Tumor cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of bortezomib. The cells were incubated with NHS (1:4) washed and then stained with polyclonal goat α human fH primary antibody (1:500), followed by Alexa Fluor 488-Labeled rb α gt IgG (1:800). Goat pre-immune serum was used as a control. The data are presented as mean values ± SD for fH-binding (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.001$ (***) whilst (ns) indicates non-significant differences. One-way ANOVA, followed by *Dunn's Multiple Comparison* test was used for statistical analysis.

3.4.3. Cell lysis by CDC

71% of untreated BT474 cells were lysed at 24h by CDC. 75.8%, 65.3% and 79.5% of 5nM, 10nM and 20nM bortezomib-treated cells were lysed, respectively ($p > 0.05$). In presence of mCRP neutralising Abs, 74.7% of untreated cells were lysed. 72.4% and 72.8% of the cells were lysed at 5nM and 10nM bortezomib, respectively, whereas 89.5% of 20nM bortezomib-treated cells were lysed with a significant increase by 14.9% ($p < 0.05$; Figure 14a).

At 48h, 96.6% of untreated BT474 cells were lysed. 65.8%, 72.2% and 69.1% of the cells were lysed with a significant decrease by 30.8%, 24.4% and 27.5% at 5nM, 10nM and 20nM bortezomib, respectively ($p < 0.001$). In presence of mCRP neutralising Abs, 83.8% of untreated cells were lysed. 70.2%, 58.2% and 63.5% of bortezomib-treated cells were lysed with a significant decrease by 13.6%, 25.6% and 63.5% at 5nM, 10nM and 20nM drug concentration, respectively ($p < 0.001$; Figure 14b).

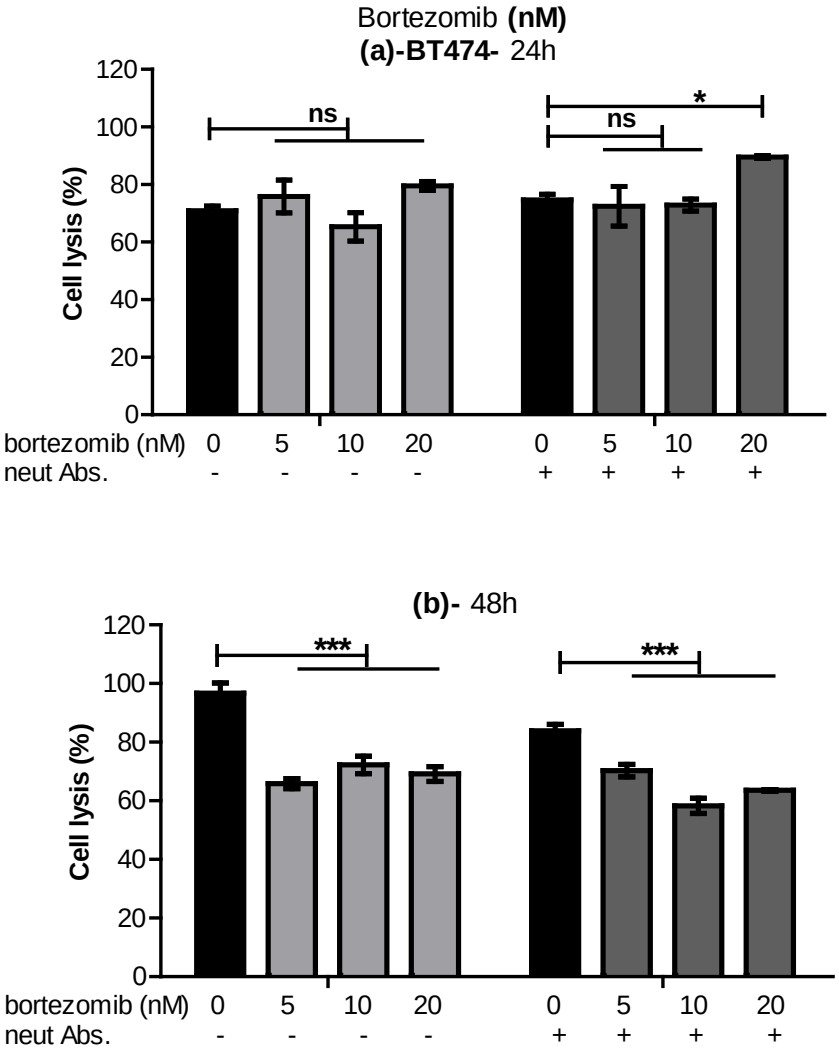
73.3% of untreated SKBR-3 cells were lysed at 24h by CDC. 67.3% of 5nM bortezomib-treated cells were lysed with a reduction by 6% ($p > 0.05$), 45.7% of 10nM bortezomib-treated cells were lysed with a reduction by 27.6% ($p < 0.01$) and 52.3% of 20nM bortezomib-treated cells were lysed with a reduction by 21% ($p < 0.01$). In presence of mCRP neutralising Abs, 76% of untreated cells were lysed. 71.4%, 54% and 60.4% of bortezomib-treated cells were lysed with a reduction by 4.6% at 5nM ($p > 0.05$), but it was with a significant reduction by 22% and 15.6% at 10nM and 20nM drug concentration, respectively ($p < 0.01$; Figure 14c).

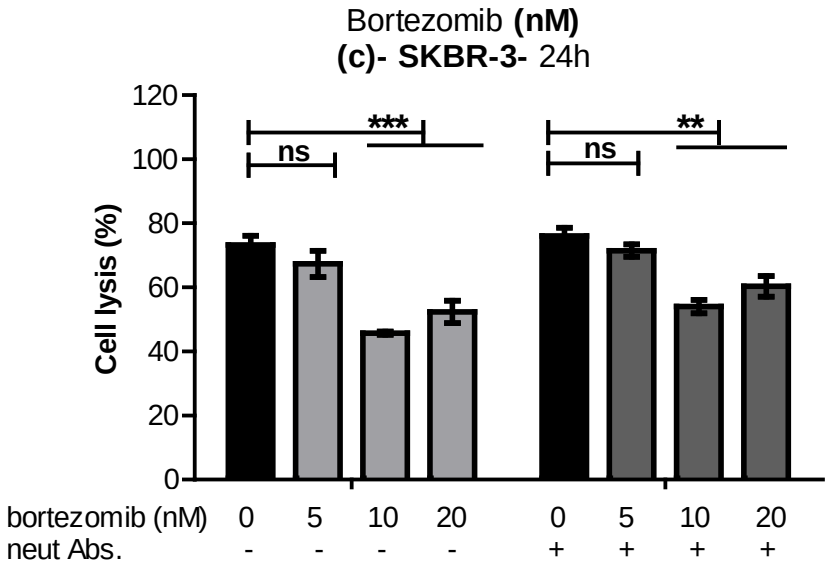
At 48h, 66.1% of untreated SKBR-3 cells were lysed. 76.5%, 95.4% and 76.6% of the cells were lysed with an increase by 10.4% ($p > 0.05$) at 5nM bortezomib, with a significant increase by 29.3% ($p < 0.001$) at 10nM bortezomib and with an increase by 10.5% ($p > 0.05$) at 20nM bortezomib. In presence of mCRP neutralizing Abs, 73.6% of untreated cells were lysed. 86.4%, 104.4% and 77.6% of bortezomib-treated cells were lysed with an increase by 12.8% at 5nM ($p > 0.05$), with a significant increase by 30.8% ($p < 0.001$) at 10nM and with an increase by 4% at 20nM ($p > 0.05$; Figure 14d).

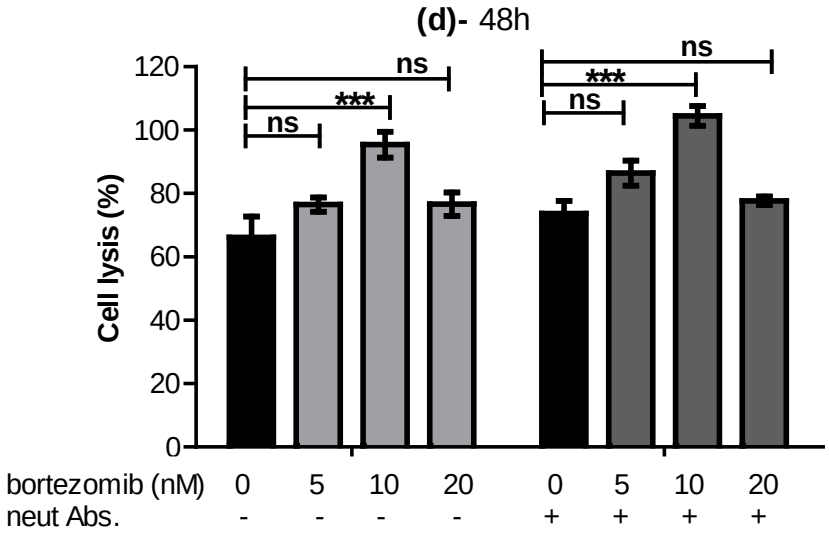
70.1% of untreated Raji cells were lysed at 24h by CDC. 48.1% and 36.7% of bortezomib-treated cells were lysed with a significant reduction by

22% at 5nM ($p < 0.01$) and with a significant reduction by 33.4% at 20nM ($p < 0.001$). 36.8% of 10nM bortezomib-treated cells were lysed with a reduction by 6.4% ($p > 0.05$). In presence of mCRP neutralising Abs, 63.7% of untreated cells were lysed. 57.8%, 59.9% and 46.8% of bortezomib-treated cells were lysed with a reduction by 5.9% at 5nM, a reduction by 3.8% at 10nM ($p > 0.05$) whereas a significant reduction by 16.9% was noticed at 20nM bortezomib ($p < 0.05$; Figure 14e).

At 48h, 52.7% of untreated Raji cells were lysed. 50.7%, 63.4% and 52.6% of the cells were lysed with a reduction by 2% at 5nM bortezomib ($p > 0.05$), with a significant increase by 10.7% at 10nM bortezomib ($p > 0.01$). In presence of mCRP neutralising Abs, 54.7% of untreated cells were lysed. 59.6%, 72% and 61.6% of bortezomib-treated cells were lysed with an increase by 4.9% at 5nM ($p > 0.05$), with a significant increase by 17.3% at 10nM ($p > 0.01$) and with an increase by 6.9% at 20nM ($p > 0.05$; Figure 14f).


Bortezomib (nM)
(a)-BT474- 24h
Cell lysis (%)
120
100
80
60
40
20
0
ns
ns
*
bortezomib (nM) 0 5 10 20 0 5 10 20
neut Abs. - - - - + + + +
(b)- 48h
Cell lysis (%)
***
***
bortezomib (nM) 0 5 10 20 0 5 10 20
neut Abs. - - - - + + + +


Bortezomib (nM)
(c)- SKBR-3- 24h
Cell lysis (%)
120
100
80
60
40
20
0
ns
***
ns
**
bortezomib (nM) 0 5 10 20 0 5 10 20
neut Abs. - - - - + + + +


(d)- 48h
Cell lysis (%)
120
100
80
60
40
20
0
ns
***
ns
ns
***
ns
bortezomib (nM) 0 5 10 20 0 5 10 20
neut Abs. - - - - + + + +

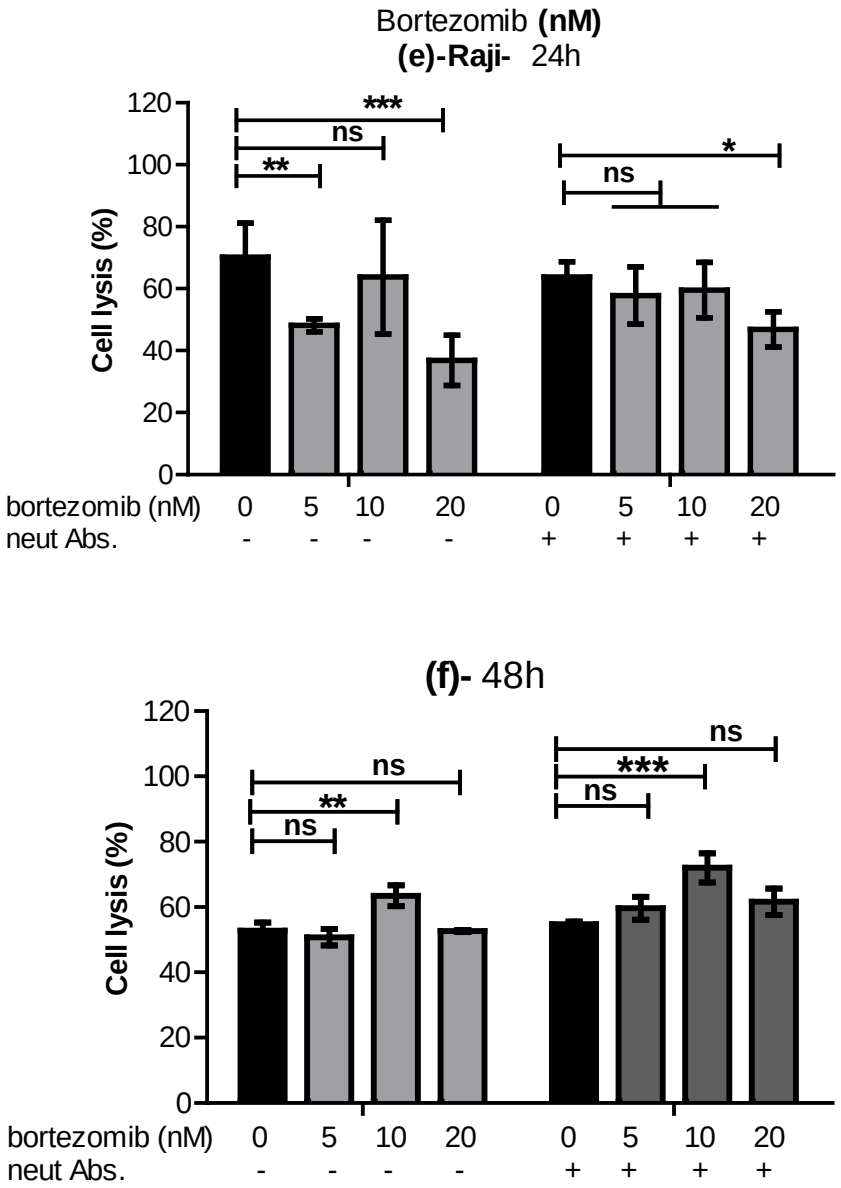


Fig. 14: CDC of different bortezomib-treated cancer cell lines.
(a&b) CDC of BT474 cells at 24& 48h. **(c&d)** CDC of SKBR-3 cells at 24 & 48h. **(e&f)** CDC of Raji cells at 24h & 48h. Tumor cells ($5x10^5$/well) were allowed to grow for 24h and 48h either without treatment or with indicated concentrations of bortezomib. The cells were labelled with ^{51}Cr and then incubated with α tumor antibodies (1:50) or with Rituximab (10µg/ml) in absence or presence of ms α hum mCRP neutralising antibodies (2µg/ml). Human serum (25%) was used as a complement source. The data are presented as mean values ± SD for CDC (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.01$ (**), $p < 0.001$ (***) whilst (ns) indicates non-significant differences). Two-way ANOVA, followed by Bonferroni *post hoc* test was used for statistical analysis

3.4.4. **Opsonisation with iC3b**

50.5% of untreated BT474 cells were opsonised with iC3b at 48h. 42.7%, 59.6% and 58.7% of bortezomib-treated cells stained positive for iC3b with a significant reduction by 7.8% at 5nM ($p < 0.05$), with a significant increase by 9.1% and 8.2% at 10nM and 20nM drug concentration, respectively ($p < 0.05$; Figure 15a).

53.9% of the untreated SKBR-3, cells stained positive for iC3b at 48h. 66.9%, 67.4% and 73.4% of the cells were iC3b positive with a significant increase by 13% at 5nM ($p < 0.05$), 13.5% at 10nM ($p < 0.05$) and by 19.5 % at 20nM drug concentration ($p < 0.01$; Figure 15b).

10.8% of untreated Raji cells were iC3 positive at 48h. 13.4%, 12.6% and 15.3% of bortezomib-treated cells were opsonised with iC3b at 5nM, 10nM and 20nM, respectively ($p > 0.05$; Figure 15c).

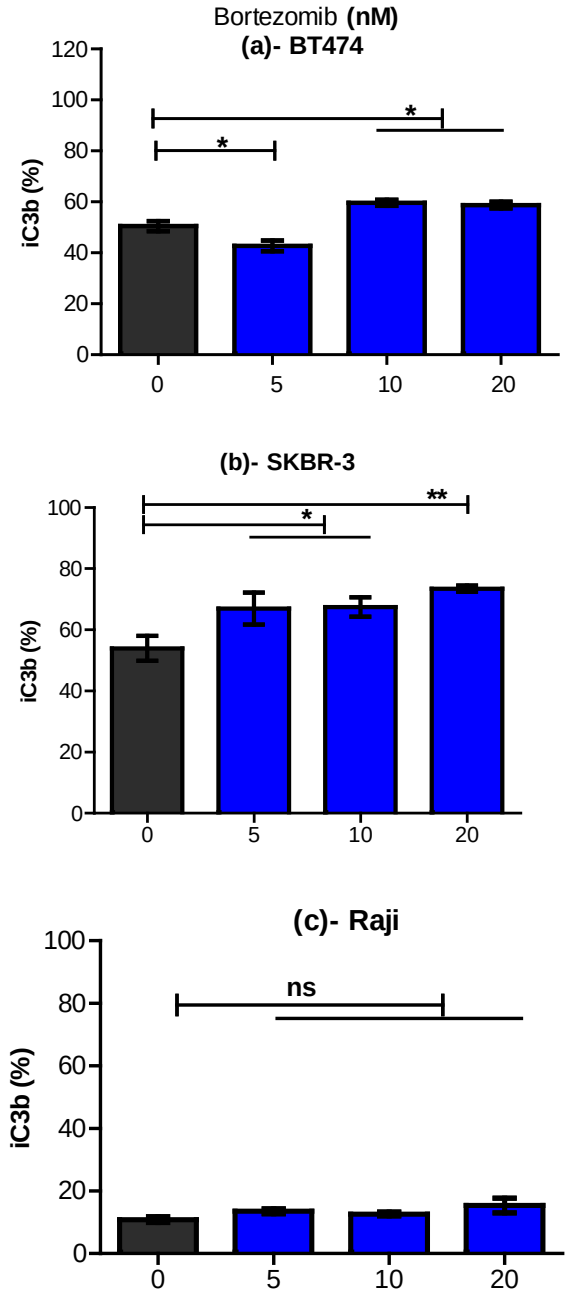


Fig. 15: Opsonisation of different bortezomib-treated cancer cell lines with iC3b. (a) BT474 cells. **(b)** SKBR-3 cells. **(c)** Raji cells. Tumor cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of bortezomib. The cells were incubated with specific polyclonal α tumor antibodies (1:50) or rituximab (10 µg/ml), followed by adding either C8-depleted or heat-inactivated serum as a complement source. The cells were stained with monoclonal ms α human iC3b primary antibody (10µg/ml), followed by goat α ms IgG-FITC (1:50). The data are presented as mean values ± SD for iC3b positive cells (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.01$ (**) whilst (ns) indicates non-significant differences. One-way ANOVA, followed by *Dunn's Multiple Comparison* test was used for statistical analysis.

3.5. Impact of fludarabine on Raji cells

3.5.1. Expression of CD46, CD55 and CD59

20.8% of untreated Raji cells were CD46 positive at 24h. 19.6%, 19.8% and 19.6% of the cells were positive for this regulator at 1μM, 3μM and 5μM fludarabine, respectively. 22.8% of untreated cells were CD55 positive. 23%, 22% and 22.4% of the cells were positive for this regulator at 1μM, 3μM and 5μM fludarabine, respectively. 16.5% of untreated cells stained positive for CD59. 16.7%, 16.2% and 16.3% of fludarabine-treated cells were positive for this regulator at 1μM, 3μM and 5μM fludarabine, respectively (Figure 16a).

At 48h, 35.5% of untreated Raji cells were CD46 positive. 28%, 27.3% and 28.5% of the cells stained positive for this inhibitor with a significant reduction by 7.5%, 8.2% and 7% at 1μM, 3μM and 5μM fludarabine, respectively ($p < 0.001$). 20.1% of untreated cells were CD55 positive. 20.2%, 20.3% and 21.1% of fludarabine-treated cells were positive for this regulator, at 1μM, 3μM and 5μM, respectively. 16.7% of untreated cells were CD59 positive. 24.2%, 22.5% and 22.2% of the cells stained positive for this mCRP with a significant increase by 7.5%, 5.8% and 5.5% at 1μM, 3μM and 5μM drug concentration, respectively ($p < 0.001$; Figure 16b).

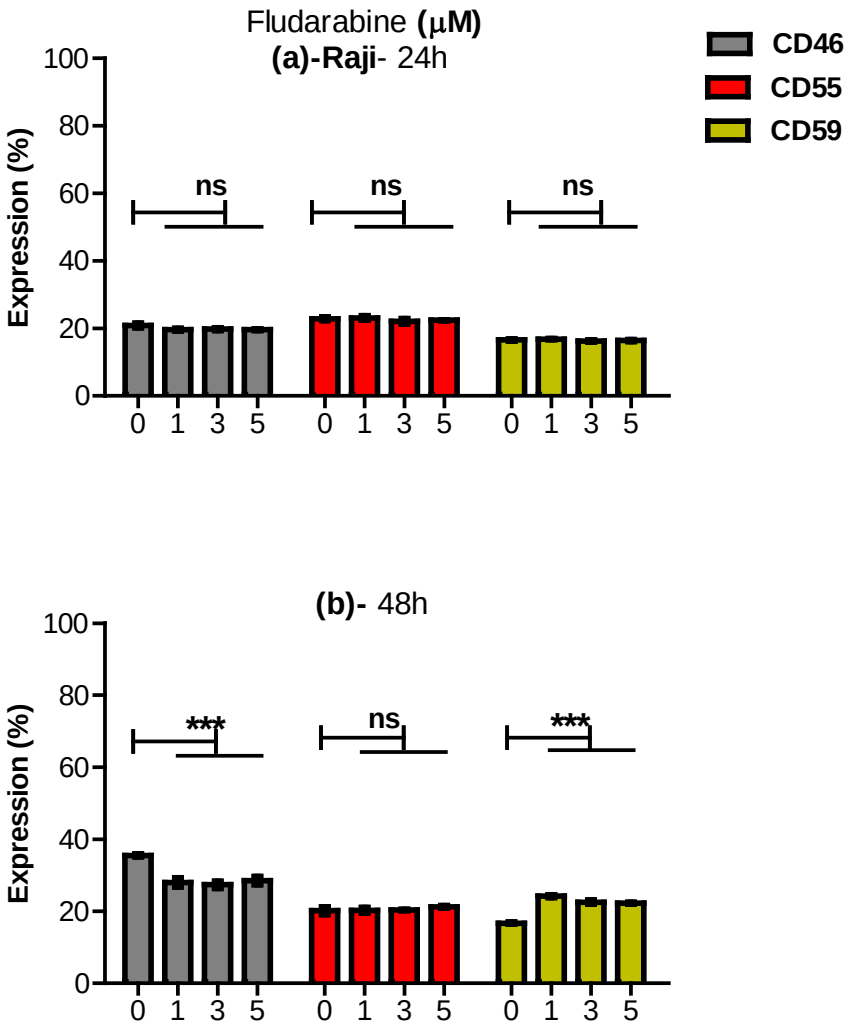


Fig. 16: Expression of CD46, CD55 and CD59 by fludarabine-treated Raji cells. **(a)** Raji cells at 24h. **(b)** Raji cells at 48h. Tumor cells ($5x10^5$/well) were allowed to grow for 24h and 48h either without treatment or with indicated drug concentrations. The cells were stained with ms IgG1 isotype control or with monoclonal ms α human CD59, CD55 and CD46 primary antibodies (10μg/ml followed by goat α ms IgG-FITC (1:50). The data are presented as mean values ± SD for the mCRP expression (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) whilst (ns) indicates non-significant differences. Two-way ANOVA, followed by Bonferroni *post hoc* test was used for statistical analysis.

3.5.2. **Complement fH binding**

13.2% of untreated Raji cells were positive for fH binding at 48h. 25.7%, 40.8% and 35.6% of fludarabine-treated cells stained positive for fH with a significant increase by 12.5% at 1µM ($p < 0.05$), by 27.6% and 22.4% at 3µM and 5µM fludarabine, respectively ($p < 0.001$; Figure 17).

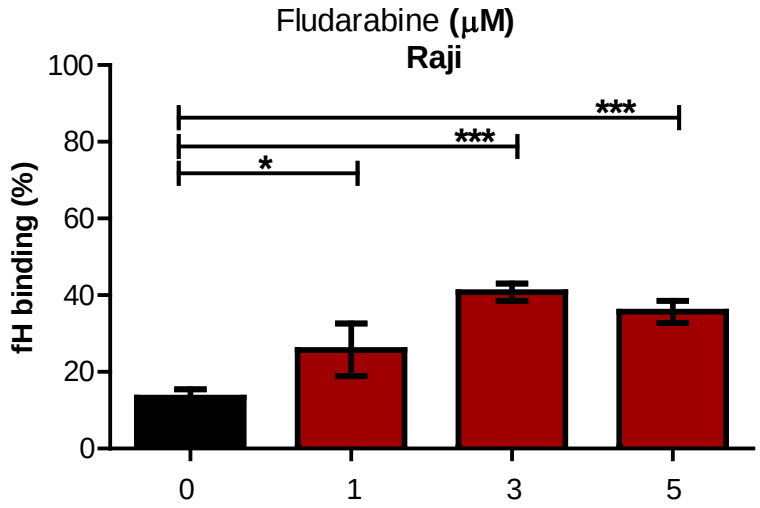


Fig. 17: Complement fH binding to fludarabine-treated Raji cells.
Tumor cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of fludarabine for 48h. The cells were incubated with NHS (1:4) washed and then stained with polyclonal goat α human fH primary antibody (1:500), followed by Alexa Fluor 488-Labeled rb α gt IgG (1:800). Goat pre-immune serum was used as a control. The data are presented as mean values ± SD for fH positive cells (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.001$ (***) whilst (ns) indicates non-significant differences. One-way ANOVA, followed by *Dunn's Multiple Comparison* test was used for statistical analysis.

3.5.3. **Cell lysis by CDC**

57.7% of untreated Raji cells were lysed at 24h by CDC. 25.4%, 14.5% and 5.5% of fludarabine-treated cells were lysed with a significant reduction by 32.3%, 43.2% and 52.2% at 1μM, 3μM and 5μM, respectively ($p < 0.001$). In presence of mCRP neutralising Abs, 41.3% of untreated cells were lysed. 33.3%, 21.4% and 9.7% of the cells were lysed with a reduction by 8% at 1μM ($p < 0.01$), 19.9% and 31.6% at 3μM and 5μM fludarabine, respectively ($p < 0.001$; Figure 18a).

At 48h, 45.4% of untreated Raji cells were lysed. 6.7%, 3.5% and 2.5% of the cells were lysed with a significant reduction by 38.7%, 41.9% and 43.1% at 1μM, 3μM and 5μM fludarabine, respectively ($p < 0.001$). In presence of mCRP neutralising Abs, 38.5% of untreated cells were lysed. 8.7%, 3.7% and 1.7% of the cells were lysed with a significant reduction by 29.8%, 34.8% and 36.8% at 1μM, 3μM and 5μM fludarabine, respectively ($P < 0.001$; Figure 18b).

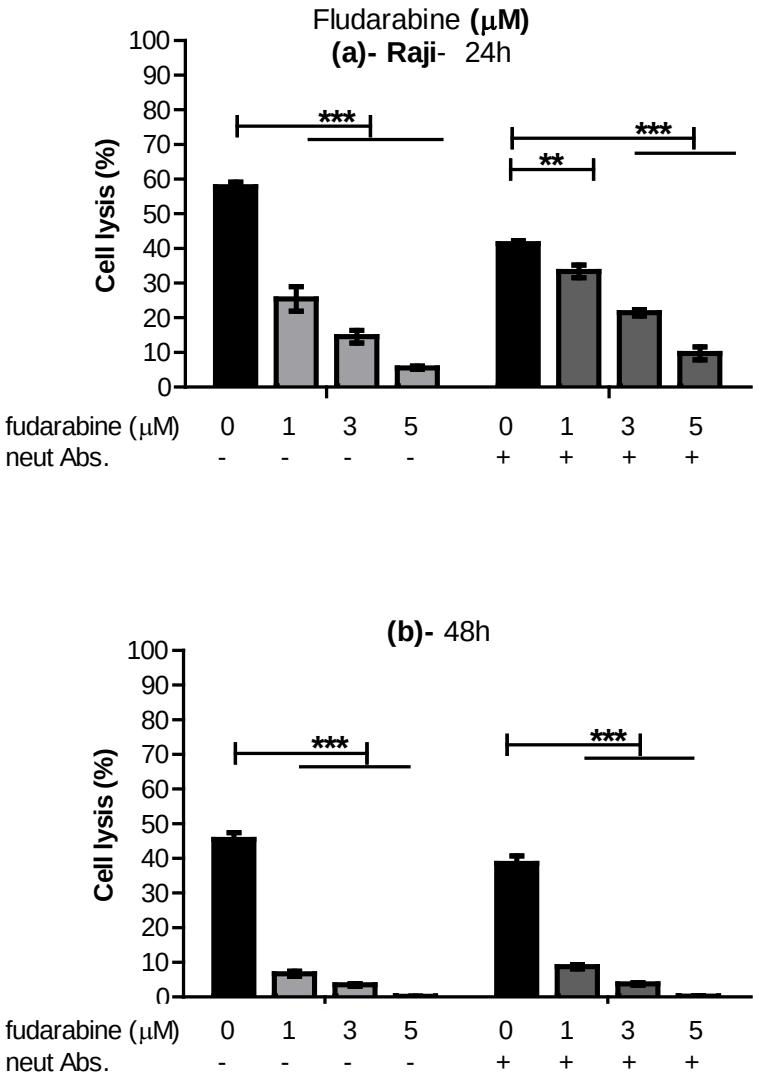


Fig. 18: Cell lysis of fludarabine- treated Raji cells by CDC.

(a) CDC at 24h. **(b)** CDC at 48h.Tumor cells ($5x10^5$/well) were allowed to grow for 24h and 48h either without treatment or with indicated fludarabine concentrations. The cells were labelled with ^{51}Cr and then incubated with Rituximab (10μg/ml) in absence or presence of α mCRP neutralising antibodies (2μg/ml). Human serum (25%) was used as a complement source. The data are presented as mean values ± SD for CDC (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.01$ (**), $p < 0.001$ (***) whilst (ns) indicates non-significant differences. Two-way ANOVA, followed by Bonferroni *post hoc* test was used for statistical analysis

3.5.4. **Opsonisation with iC3b**

10.8% of untreated Raji cells were opsonised with iC3b at 48h. 19.3%, 18.5% and 24% of fludarabine-treated cells were iC3b positive with an increase by 8.5% at 1µM ($p < 0.01$), 7.7% at 3µM ($p < 0.05$) and 13.2% at 5µM ($p < 0.001$; Figure 19).

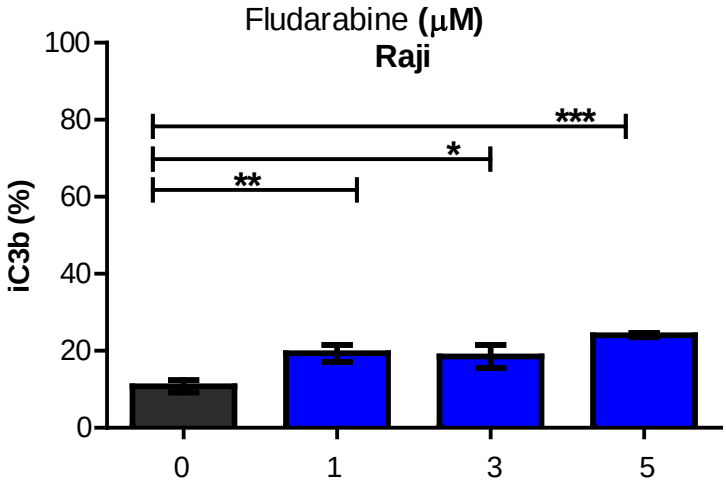


Fig. 19: Opsonisation of fludarabine-treated Raji cells with iC3b. Tumor cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated fludarabine concentrations. The cells were incubated with Rituximab (10µg/ml), followed by either C8-depleted or heat-inactivated serum as a complement source. And then, the cells were stained with monoclonal ms α human iC3b primary antibody (10µg/ml), followed by goat α ms IgG-FITC (1:50). The data are presented as mean values ± SD for iC3b positive cells (in %). In three independent experiments, three biological replicates at each drug concentration were analyzed. Significant differences are indicated by asterisks, $p < 0.01$ (**) whilst (ns) indicates non-significant differences. One-way ANOVA, followed by *Dunn's Multiple Comparison* test was used for statistical analysis.

3.6. Influence of anti-cancer drugs on the secretion of soluble complement regulatory proteins by cancer cells

In only one out of four independent experiments, secretion of complement factor H (fH) into cell culture supernatant of doxorubicin-treated SKBR3 cells was observed (data not presented). We assume that the level of secretion of this complement regulator was either under the detection limit of our assay or our cell lines could did not secrete this regulator into the surrounding environment.

3.7. Summarizing tables of results

3.7.1. Impact of doxorubicin on breast cancer cell lines on the expression of mCRPs (CD46, CD55 and CD59), cell lysis by CDC, fH binding and opsonisation with iC3b (mean values and SD of 3 independent experiments are presented).

BT474 cell line									
		24h					48h		
		Doxorubicin (μM)							
	Conc.	**0**	**2**	**5**	**10**		**0**	**2**	**5**	**10**
	Analyzed markers									
	CD46 (%)	33.7 32.0 33.4	45.3 44.0 44.6	33.7 35.6 36.2	35.7 36.1 36.5		37.9 32.6 38.1	24.8 25.0 31.3	28.3 30.0 33.2	31.0 34.0 40.7
Mean		**33.0**	**44.6**	**35.1**	**36.1**		**36.2**	**27.0**	**30.5**	**35.2**
SD		0.9	0.6	1.3	0.4		3.1	3.6	2.4	4.9
	CD55 (%)	17.7 19.1 19.5	22.8 20.9 22.1	30.8 31.6 31.4	30.9 34.2 34.0		16.3 12.7 15.3	19.7 19.0 18.1	25.1 25.6 28.4	35.1 33.8 35.2
Mean		**18.7**	**21.9**	**31.2**	**33.0**		**14.7**	**18.9**	**26.3**	**34.7**
SD		0.9	0.9	0.4	1.8		1.8	0.80	1.7	0.7
	CD59 (%)	27.3 29.1 31.0	41.1 43.0 44.9	35.6 37.9 37.6	36.6 36.6 36.9		43.3 45.1 50.0	51.5 48.3 54.2	59.7 59.3 45.8	57.6 55.6 53.8
Mean		**29.1**	**43.0**	**37.0**	**36.7**		**46.1**	**51.3**	**54.9**	**55.6**
SD		1.8	1.9	1.2	0.1		3.4	2.9	7.9	1.9
	CDC (%)	88.8 81.3 69.9	36.0 34.4 33.7	26.2 26.8 25.3	29.8 29.1 26.8		95.4 80.2 100.4	25.5 22.1 28.0	16.1 19.2 16.5	20.5 18.5 22.2
Mean		**80.0**	**34.7**	**26.1**	**28.5**		**92.0**	**25.2**	**17.2**	**20.4**
SD		7.7	0.9	0.6	1.2		8.5	2.4	1.3	1.5
	CDC (%) +mCRP neutral.	84.7 79.4 81.0	37.2 34.1 36.3	32.0 30.3 31.5	26.4 28.6 29.4		74.9 77.5 96.6	25.0 21.7 23.7	18.5 16.9 18.6	19.5 21.8 22.6
Mean		**81.7**	**35.8**	**31.2**	**28.1**		**83.0**	**23.4**	**18.0**	**21.3**
SD		2.7	1.5	0.8	1.5		11.8	1.6	0.9	1.6
	fH-binding (%)						19.0 19.0 23.0	48.0 66.0 69.0	118.0 117.0 64.0	84.0 83.0 86.0
Mean							**20.3**	**61.0**	**99.6**	**84.3**
SD							2.3	11.3	30.8	1.5
	iC3b (%)						44.6 52.3 51.9 53.2	69.1 61.2 67.7 60.6	60.7 41.0 58.0 60.9	54.2 62.8 62.2 63.1

Mean							**50.5**	**64.6**	**55.1**	**60.5**
SD							3.97	4.37	9.52	4.26
SKBR-3 cell line										
24h						48h				
Doxorubicin (µM)										
	Conc.	**0**	**2**	**5**	**10**		**0**	**2**	**5**	**10**
	Analyzed markers									
	CD46 (%)	35.9 48.3 39.8	59.2 62.4 58.3	62.8 61.7 62.3	74.5 70.5 72.0		46.5 48.0 45.0	32.6 33.0 33.4	44.1 46.4 48.3	52.1 52.6 52.3
Mean		**41.3**	**59.9**	**62.2**	**72.3**		**46.5**	**33.4**	**46.2**	**52.3**
SD		6.3	2.1	0.5	2.0		1.5	0.4	2.1	0.2
	CD55 (%)	26.8 25.6 25.2	35.6 34.6 33.3	43.4 44.2 44.3	60.2 58.6 63.2		57.7 57.7 57.1	38.3 35.9 39.8	49.1 49.4 47.9	52.8 52.0 52.8
Mean		**25.8**	**34.5**	**43.9**	**60.6**		**57.5**	**38.0**	**48.8**	**52.5**
SD		0.8	1.1	0.4	2.3		0.3	1.9	0.7	0.4
	CD59 (%)	67.2 67.8 66.3	64.5 74.3 81.9	69.9 72.1 80.6	80.6 79.6 79.3		68.8 69.7 68.7	66.2 68.1 66.2	68.8 69.1 73.8	69.4 70.1 70.0
Mean		**67.1**	**73.5**	**74.2**	**79.8**		**69.0**	**66.8**	**70.5**	**69.8**
SD		0.7	8.7	5.6	0.6		0.5	1.0	2.8	0.3
	CDC (%)	67.7 49.4 54.3	19.6 21.1 20.1	28.6 29.7 27.3	33.1 33.9 38.2		53.0 61.1 50.5	37.3 39.9 48.5	59.1 57.5 68.7	52.7 54.0 59.9
Mean		**57.1**	**20.2**	**28.5**	**35.0**		**54.8**	**41.9**	**61.7**	**55.5**
SD		7.7	0.62	0.98	2.2		4.5	4.7	4.9	3.1
	CDC (%) +mCRPs neutral.	65.4 66.5 48.7	35.1 31.6 30.5	28.6 29.7 43.4	33.1 33.9 38.2		54.6 48.2 53.9	39 37.8 36.7	55.5 57 58.9	66.9 61 62
Mean		**60.2**	**32.4**	**33.9**	**35.7**		**52.2**	**37.8**	**57.1**	**63.3**
SD		9.9	2.4	8.2	2.7		3.5	1.1	1.7	3.1
	fH binding (%)						36.1 36.4 35.7	37.2 36.4 38.1	41.7 41.3 41.3	33,4 31.7 32.6
Mean							**36.0**	**37.2**	**41.4**	**32.5**
SD							0.3	0.8	0.2	0.8
	iC3b (%)						38.4 43.8 47.9	47.7 51.2 47.4	54.2 61.2 67.5	62.0 61.8 59.0
Mean							**43.3**	**48.7**	**60.9**	**60.9**
SD							4.76	2.11	6.6	1.6

BT474 cell line										
		24h					48h			
		Doxorubicin (µM)								
	Conc.	**0**	**2**	**5**	**10**		**0**	**2**	**5**	**10**
	Analyzed markers									
	CD46 (%)	33.7 32.0 33.4	45.3 44.0 44.6	33.7 35.6 36.2	35.7 36.1 36.5		37.9 32.6 38.1	24.8 25.0 31.3	28.3 30.0 33.2	31.0 34.0 40.7
Mean		**33.0**	**44.6**	**35.1**	**36.1**		**36.2**	**27.0**	**30.5**	**35.2**
SD		0.9	0.6	1.3	0.4		3.1	3.6	2.4	4.9
	CD55 (%)	17.7 19.1 19.5	22.8 20.9 22.1	30.8 31.6 31.4	30.9 34.2 34.0		16.3 12.7 15.3	19.7 19.0 18.1	25.1 25.6 28.4	35.1 33.8 35.2
Mean		**18.7**	**21.9**	**31.2**	**33.0**		**14.7**	**18.9**	**26.3**	**34.7**
SD		0.9	0.9	0.4	1.8		1.8	0.80	1.7	0.7
	CD59 (%)	27.3 29.1 31.0	41.1 43.0 44.9	35.6 37.9 37.6	36.6 36.6 36.9		43.3 45.1 50.0	51.5 48.3 54.2	59.7 59.3 45.8	57.6 55.6 53.8
Mean		**29.1**	**43.0**	**37.0**	**36.7**		**46.1**	**51.3**	**54.9**	**55.6**
SD		1.8	1.9	1.2	0.1		3.4	2.9	7.9	1.9
	CDC (%)	88.8 81.3 69.9	36.0 34.4 33.7	26.2 26.8 25.3	29.8 29.1 26.8		95.4 80.2 100.4	25.5 22.1 28.0	16.1 19.2 16.5	20.5 18.5 22.2
Mean		**80.0**	**34.7**	**26.1**	**28.5**		**92.0**	**25.2**	**17.2**	**20.4**
SD		7.7	0.9	0.6	1.2		8.5	2.4	1.3	1.5
	CDC (%) +mCRPs neutral.	84.7 79.4 81.0	37.2 34.1 36.3	32.0 30.3 31.5	26.4 28.6 29.4		74.9 77.5 96.6	25.0 21.7 23.7	18.5 16.9 18.6	19.5 21.8 22.6
Mean		**81.7**	**35.8**	**31.2**	**28.1**		**83.0**	**23.4**	**18.0**	**21.3**
SD		2.7	1.5	0.8	1.5		11.8	1.6	0.9	1.6
	fH binding (%)						19.0 19.0 23.0	48.0 66.0 69.0	118.0 117.0 64.0	84.0 83.0 86.0
Mean							**20.3**	**61.0**	**99.6**	**84.3**
SD							2.3	11.3	30.8	1.5
	iC3b (%)						44.6 52.3 51.9 53.2	69.1 61.2 67.7 60.6	60.7 41.0 58.0 60.9	54.2 62.8 62.2 63.1
Mean							**50.5**	**64.6**	**55.1**	**60.5**
SD							3.97	4.37	9.52	4.26

SKBR-3 cell line										
		24h					48h			
		Doxorubicin (μM)								
	Conc.	**0**	**2**	**5**	**10**		**0**	**2**	**5**	**10**
	Analyzed markers									
	CD46 (%)	35.9 48.3 39.8	59.2 62.4 58.3	62.8 61.7 62.3	74.5 70.5 72.0		46.5 48.0 45.0	32.6 33.0 33.4	44.1 46.4 48.3	52.1 52.6 52.3
Mean		**41.3**	**59.9**	**62.2**	**72.3**		**46.5**	**33.4**	**46.2**	**52.3**
SD		6.3	2.1	0.5	2.0		1.5	0.4	2.1	0.2
	CD55 (%)	26.8 25.6 25.2	35.6 34.6 33.3	43.4 44.2 44.3	60.2 58.6 63.2		57.7 57.7 57.1	38.3 35.9 39.8	49.1 49.4 47.9	52.8 52.0 52.8
Mean		**25.8**	**34.5**	**43.9**	**60.6**		**57.5**	**38.0**	**48.8**	**52.5**
SD		0.8	1.1	0.4	2.3		0.3	1.9	0.7	0.4
	CD59 (%)	67.2 67.8 66.3	64.5 74.3 81.9	69.9 72.1 80.6	80.6 79.6 79.3		68.8 69.7 68.7	66.2 68.1 66.2	68.8 69.1 73.8	69.4 70.1 70.0
Mean		**67.1**	**73.5**	**74.2**	**79.8**		**69.0**	**66.8**	**70.5**	**69.8**
SD		0.7	8.7	5.6	0.6		0.5	1.0	2.8	0.3
	CDC (%)	67.7 49.4 54.3	19.6 21.1 20.1	28.6 29.7 27.3	33.1 33.9 38.2		53.0 61.1 50.5	37.3 39.9 48.5	59.1 57.5 68.7	52.7 54.0 59.9
Mean		**57.1**	**20.2**	**28.5**	**35.0**		**54.8**	**41.9**	**61.7**	**55.5**
SD		7.7	0.62	0.98	2.2		4.5	4.7	4.9	3.1
	CDC (%) +mCRPs neutral.	65.4 66.5 48.7	35.1 31.6 30.5	28.6 29.7 43.4	33.1 33.9 38.2		54.6 48.2 53.9	39 37.8 36.7	55.5 57 58.9	66.9 61 62
Mean		60.2	32.4	33.9	35.7		52.2	37.8	57.1	63.3
SD		9.9	2.4	8.2	2.7		3.5	1.1	1.7	3.1
	fH binding (%)						36.1 36.4 35.7	37.2 36.4 38.1	41.7 41.3 41.3	33,4 31.7 32.6
Mean							**36.0**	**37.2**	**41.4**	**32.5**
SD							0.3	0.8	0.2	0.8
	iC3b (%)						38.4 43.8 47.9	47.7 51.2 47.4	54.2 61.2 67.5	62.0 61.8 59.0
Mean							**43.3**	**48.7**	**60.9**	**60.9**
SD							4.76	2.11	6.6	1.6

3.7.2. Impact of taxol on breast cancer cell lines on the expression of mCRPs (CD46, CD55 and CD59), cell lysis by CDC, fH binding and opsonisation with iC3b (mean values and SD of 3 independent experiments are presented).

BT474 cell line										
		24h					48h			
		Taxol (nM)								
	Conc.	**0**	**5**	**10**	**20**		**0**	**5**	**10**	**20**
	Analyzed markers									
	CD64 (%)	58.0 64.6 60.4	53.5 56.9 55.9	41.3 47.7 43.5	35.2 34.5 36.5		68.9 68.9 71.0	68.0 63.7 72.7	70.2 80.1 32.5	78.1 84.6 82.2
Mean		**61.0**	**55.4**	**44.1**	**35.4**		**69.6**	**68.1**	**60.9**	**81.6**
SD		3.3	1.7	3.2	1.0		1.2	4.5	25.1	3.2
	CD55 (%)	21.1 27.6 29.9	22.7 24.9 25.4	26.5 21.8 23.5	25.0 27.0 27.2		19.4 19.4 41.1	34.2 33.2 47.4	32.3 33.3 32.5	37.4 36.0 72.2
Mean		**26.2**	**24.3**	**23.9**	**26.4**		**26.6**	**38.2**	**32.7**	**48.5**
SD		4.5	1.4	2.3	1.2		12.5	7.9	0.5	20.5
	CD59 (%)	66.1 63.0 64.6	60.5 56.5 61.0	53.8 51.6 50.4	45.9 46.2 47.0		74.8 67.2 69.3	70.3 69.7 71.2	65.3 64.6 75.5	72.4 64.2 69.1
Mean		**64.5**	**59.3**	**51.9**	**46.3**		**70.4**	**70.4**	**68.4**	**68.5**
SD		1.5	2.4	1.7	0.5		3.9	0.7	6.1	4.1
	CDC (%)	78.9 67.9 71.2	61.3 60.4 64.7	62.0 57.3 63.5	66.1 70.2 70.0		84.4 85.7 81.8	65.6 64.1 71.8	78.8 74.0 74.3	50.7 48.9 47.5
Mean		**72.6**	**62.1**	**60.9**	**68.7**		**83.9**	**67.1**	**75.7**	**49.0**
SD		5.6	2.2	3.2	2.3		1.9	4.0	2.6	1.6
	CDC (%) +mCRPs neutral.	50.0 52.6 60.5	53.1 49.3 59.2	46.7 50.9 83.1	48.1 58.0 78.8		47.9 45.4 49.3	56.0 53.0 57.3	74.4 69.4 72.6	34.5 36.6 45.4
Mean		**54.3**	**53.8**	**60.2**	**61.6**		**47.5**	**55.4**	**72.1**	**38.8**
SD		5.4	4.9	19.9	15.6		1.9	2.2	2.5	5.7
	fH binding (%)						24.2 25.8 25.1 25.0 25.6	23.2 23.3 23.7 24.6 23.7	26.4 26.3 29.1 29.0 27.7	33.2 31.1 33.8 33.2 33.0
Mean							**25.1**	**23.7**	**27.7**	**32.8**
SD							0.6	0.5	1.3	1.0
	iC3b (%)						44.6 52.3 51.9 53.2	77.1 81.7 81.8 85.3	82.7 78.8 80.0 78.9	76.5 78.4 78.1 72.5

Mean							**50.5**	**81.4**	**80.1**	**76.3**
SD							3.9	3.3	1.8	2.7
SKBR-3 cell line										
24h							48h			
Taxol (nM)										
	Conc.	**0**	**5**	**10**	**20**		**0**	**5**	**10**	**20**
	Analyzed markers									
	CD46 (%)	16.0 17.7 17.5	42.3 46.4 44.4	46.5 47.5 49.0	62.5 60.0 65.9		19.8 17.6 16.7	39.6 44.8 42.3	38.5 41.3 45.1	51.4 43.7 49.0
Mean		**17.0**	**44.3**	**47.6**	**62.8**		**18.0**	**42.2**	**41.6**	**48.0**
SD		0.9	2.0	1.2	2.9		1.5	2.6	3.3	3.9
	CD55 (%)	17.3 17.5 18.1	29.9 29.6 30.1	31.9 30.3 31.6	39.9 41.2 39.8		30.5 33.0 38.8	30.7 31.9 31.8	25.4 26.6 25.3	27.8 25.5 25.4
Mean		**17.6**	**29.8**	**31.2**	**40.3**		**34.1**	**31.4**	**25.7**	**26.2**
SD		0.4	0.2	0.8	0.7		4.2	0.6	0.7	1.3
	CD59 (%)	38.2 40.5 40.1	58.0 58.5 58.3	62.0 58.8 54.1	74.3 76.7 55.2		53.2 51.1 49.6	62.4 59.9 62.1	55.2 45.8 50.6	59.4 68.9 59.7
Mean		**39.6**	**58.2**	**58.3**	**68.7**		**51.3**	**61.4**	**50.5**	**62.6**
SD		1.2	0.2	3.9	11.7		1.8	1.3	4.7	5.4
	CDC (%),	79.0 78.8 89.0	79.2 74.1 80.0	60.8 61.0 73.8	42.8 37.8 36.9		69.2 70.2 68.4	51.0 48.6 49.1	49.4 45.4 48.6	3.0 3.7 3.8
Mean		**82.2**	**77.7**	**65.2**	**39.1**		**69.2**	**49.5**	**47.8**	**3.5**
SD		5.8	3.2	7.4	3.1		0.9	1.2	2.1	0.4
	CDC (%), +mCRPs neutral.	60.9 57.5 61.4	72.8 69.9 75.1	45.4 50.0 58.7	42.8 43.4 37.7		68.7 54.1 58.1	67.0 61.6 72.2	54.5 52.1 55.2	8.9 3.1 3.7
Mean		**59.9**	**72.6**	**51.3**	**41.3**		**60.3**	**66.9**	**53.9**	**5.2**
SD		2.1	2.6	6.7	3.1		7.5	5.3	1.6	3.1
	fH binding (%)						31.5 32.9 33.5	33.1 34.1 35.5	34.9 29.2 31.9	40.7 39.4 37.3
Mean							**32.6**	**34.2**	**32.0**	**39.1**
SD							1.0	1.2	2.8	1.7
	iC3b (%)						18.5 23.3 20.3	22.8 21.2 21.6	29.6 26.8 29.9	37.6 35.2 37.7
Mean							**20.7**	**21.6**	**28.7**	**36.8**
SD							2.4	0.8	1.7	1.4

3.7.3. Impact of bortezomib on different cancer cell lines on the expression of mCRPs (CD46, CD55 and CD59), cell lysis by CDC, fH binding and opsonisation with iC3b (mean values and SD of 3 independent experiments are presented).

BT474 cell line										
		24h					48h			
		Bortezomib (nM)								
	Conc.	**0**	**5**	**10**	**20**		**0**	**5**	**10**	**20**
	Analyzed markers									
	CD46 (%)	22.2 20.5 24.5	23.1 21.7 19.7	17.1 26.8 24.1	21.4 21.9 22.2		51.0 54.4 57.9	37.7 34.3 38.5	32.6 47.5 30.0	30.3 29.4 34.6
Mean		**22.4**	**21.5**	**22.6**	**21.8**		**54.4**	**36.8**	**36.7**	**31.4**
SD		2.0	1.7	5.0	0.4		3.4	2.2	9.4	2.7
	CD55 (%)	16.1 15.7 18.0	16.0 16.0 16.1	15.5 17.1 16.3	16.3 15.9 16.6		30.5 25.3 26.8	21.3 25.0 22.1	24.0 20.3 21.6	23.8 19.0 20.1
Mean		**16.6**	**16.0**	**16.3**	**16.2**		**27.5**	**22.8**	**21.9**	**20.9**
SD		1.2	0.05	0.8	0.3		2.6	1.9	1.8	2.5
	CD59 (%)	33.0 34.7 33.1	33.1 34.7 38.9	31.0 38.9 40.1	36.1 35.2 37.9		62.3 62.8 61.1	43.3 37.6 54.7	36.3 33.1 41.2	43.5 35.0 36.4
Mean		**33.6**	**35.5**	**36.6**	**36.4**		**62.0**	**45.2**	**36.8**	**38.3**
SD		0.9	2.9	4.9	1.3		0.8	8.7	4.0	4.5
	CDC (%),	73.3 71.1 68.2	65.0 84.2 79.4	66.1 73.5 56.3	82.6 77.8 78.1		92.6 93.6 103.7	65.4 69.0 63.1	69.7 68.8 78.1	65.4 68.0 74.0
Mean		**70.8**	**76.2**	**65.3**	**79.5**		**96.6**	**65.8**	**72.2**	**69.1**
SD		2.5	9.9	8.6	2.6		6.1	2.9	5.1	4.4
	CDC (%), +mCRPs neutral.	78.0 71.2 74.8	82.3 76.0 59.1	77.0 70.0 71.5	90.0 88.6 90.1		87.6 79.9 84.1	71.6 73.1 66.1	55.6 55.8 63.4	63.7 63.8 63.0
Mean		**74.6**	**72.4**	**72.8**	**89.5**		**83.8**	**70.2**	**58.2**	**63.5**
SD		3.4	11.9	3.6	0.8		3.8	3.6	4.4	0.4
	fH binding (%)						19.0 23.0 19.0	36.0 29.0 29.0	76.0 76.0 50.0	44.0 43.0 67.0
Mean							**20.3**	**31.3**	**67.3**	**51.3**
SD							2.3	4.0	15.0	13.5
	iC3b (%)						44.6 52.3 51.9 53.2	49.0 40.3 40.3 41.3	57.1 61.1 58.3 62.1	60.9 57.2 61.1 55.9

Mean							**50.5**	**42.7**	**59.6**	**58.7**
SD							3.9	4.2	2.3	2.6
SKBR-3 cell line										
24h							48h			
Bortezomib (nM)										
	Conc.	**0**	**5**	**10**	**20**		**0**	**5**	**10**	**20**
	Analyzed markers									
	CD46 (%)	29.6 26.0 23.6	27.4 22.5 23.3	26.8 27.0 24.9	35.9 34.6 32.7		22.5 23.1 23.0	21.6 22.9 21.7	21.4 20.8 21.2	21.5 21.2 21.1
Mean		**26.4**	**24.4**	**26.2**	**34.4**		**22.8**	**22.0**	**21.1**	**21.2**
SD		3.0	2.6	1.1	1.6		0.3	0.7	0.3	0.2
	CD55 (%)	14.3 16.0 15.0	16.4 15.0 16.1	17.0 17.4 15.7	18.2 17.7 18.6		25.4 24.7 26.1	24.5 26.2 25.6	25.3 22.9 26.0	23.2 25.4 24.8
Mean		**15.1**	**15.8**	**16.7**	**18.1**		**25.4**	**25.4**	**24.7**	**24.4**
SD		0.8	0.7	0.8	0.4		0.7	0.8	1.6	1.3
	CD59 (%)	42.5 40.4 40.2	42.2 41.4 40.8	43.6 44.6 44.3	47.1 50.9 52.5		32.9 30.9 30.2	29.2 31.0 30.3	29.0 31.0 31.9	32.4 28.4 32.4
Mean		**41.0**	**41.4**	**44.1**	**50.1**		**31.3**	**30.1**	**30.6**	**31.0**
SD		1.2	0.7	0.5	2.7		1.4	0.9	1.4	2.3
	CDC (%),	78.6 72.6 68.7	74.8 66.5 60.7	45.0 46.7 45.5	59.1 50.8 47.2		75.3 69.8 53.3	73.8 74.8 80.9	102.3 88.2 95.7	84.0 72.2 73.8
Mean		**73.3**	**67.3**	**45.7**	**52.3**		**66.1**	**76.5**	**95.4**	**76.6**
SD		4.9	7.0	0.8	6.1		11.4	3.8	7.0	6.4
	CDC (%), +mCRPs neutral.	81.2 72.5 74.5	71.9 74.6 67.9	58.1 51.1 52.9	54.0 63.6 63.6		72.6 81.0 67.3	92.3 78.8 88.1	109.6 104.9 98.8	80.1 77.5 75.4
Mean		**76.0**	**71.4**	**54.0**	**60.4**		**73.6**	**86.4**	**104.4**	**77.6**
SD		4.5	3.3	3.6	5.5		6.9	6.9	5.4	2.3
	fH binding (%)						17.0 18.8 22.3	19.7 23.3 19.7	19.4 20.5 20.8	19.0 18.4 17.1
Mean							**19.3**	**20.9**	**20.2**	**18.1**
SD							2.6	2.0	0.7	0.9
	iC3b (%)						57.2	61.1	68.4	73.1

							55.2 49.4	68.4 71.3	63.9 70.0	74.6 72.6
Mean							**53.9**	**66.9**	**67.4**	**73.4**
SD							4.0	5.2	3.1	1.0
Raji cell line										
24h						48h				
Bortezomib (nM)										
	Conc.	**0**	**5**	**10**	**20**		**0**	**5**	**10**	**20**
	Analyzed markers									
	CD46 (%)	31.9 33.2 33.8	35.6 33.9 34.3	39.0 39.2 36.9	37.5 38.4 40.8		43.6 49.0 44.0	48.0 47.9 49.7	46.3 46.7 47.7	48.1 48.9 46.2
Mean		**32.9**	**34.6**	**38.3**	**38.9**		**45.5**	**48.5**	**46.9**	**47.7**
SD		0.9	0.8	1.2	1.7		3.0	1.0	0.7	1.3
	CD55 (%)	31.6 32.6 27.8	32.1 35.5 34.6	35.8 34.9 36.0	33.0 37.4 37.2		43.0 41.0 39.8	41.0 41.1 39.6	39.8 44.0 40.5	43.6 42,0 40.1
Mean		**30.6**	**34.0**	**35.5**	**35.8**		**41.2**	**40.5**	**41.4**	**41.8**
SD		2.5	1.7	0.5	2.4		1.6	0.8	2.2	2.4
	CD59 (%)	30.3 32.1 28.6	30.9 33.0 32.9	35.7 34.7 34.9	33.7 35.4 34.7		38.2 38.0 37.3	40.2 41.1 39.7	40.4 37.8 35.9	38.2 40.5 35.7
Mean		**30.3**	**32.2**	**35.1**	**38.1**		**37.8**	**40.3**	**38.0**	**38.1**
SD		1.7	1.1	0.5	0.8		0.4	0.7	2.2	2.4
	CDC (%),	59.5 69.4 81.5	45.7 49.2 49.5	43.0 67.0 80.2	32.2 32.1 46.2		49.5 50.9 52.8	50.2 46.7 55.4	66.4 57.2 66.8	53.0 52.9 52.0
Mean		**70.1**	**48.1**	**63.4**	**36.8**		**51.0**	**50.7**	**63.4**	**52.6**
SD		11.0	2.1	18.8	8.1		1.6	4.3	5.4	6.5
	CDC (%), +mCRPs neutral.	59.3 63.0 69.0	48.8 57.3 67.3	49.6 61.9 67.0	53.3 45.0 42.3		55.7 53.2 55.4	55.9 56.4 66.6	69.7 65.7 80.6	63.7 53.7 67.4
Mean		**63.7**	**57.8**	**59.5**	**46.8**		**57.7**	**59.6**	**72.0**	**61.6**
SD		4.8	9.2	8.9	5.7		1.3	6.0	7.7	7.0
	fH binding (%)						11.6 12.4 15.8	10.0 8.4 8.2	11.0 10.8 11.0	23.8 22.0 23.2
Mean							**13.2**	**8.8**	**10.9**	**23.0**
SD							2.2	0.9	0.1	0.9
	iC3b (%)						11.6 9.0 11.8	11.9 14.6 13.9	12.1 11.9 13.8	12.8 13.1 20.1
Mean							**10.8**	**13.4**	**12.6**	**15.3**
SD							1.5	1.4	1.0	4.1

3.7.4. Impact of fludarabine on Raji cells on the expression of mCRPs (CD46, CD55 and CD59), cell lysis by CDC, fH binding and opsonisation with iC3b (mean values and SD of 3 independent experiments are presented).

Raji cell line									
24h						48h			
Fludarabine (µM)									
	Conc.	**0**	**1**	**3**	**5**		**0**	**1**	**3**	**5**
	Analyzed markers									
	CD46 (%)	19.7 22.0 20.9	19.2 20.4 19.3	19.0 20.4 20.0	19.5 20.0 19.3		35.0 36.2 35.3	30.3 26.0 27.7	25.4 28.0 28.7	26.3 29.0 30.2
Mean		**20.8**	**19.6**	**19.8**	**19.6**		**35.5**	**28.0**	**27.3**	**28.5**
SD		1.1	0.6	0.7	0.3		0.6	2.1	1.7	1.9
	CD55 (%)	22.0 23.8 22.7	23.4 21.8 24.0	22.1 20.7 23.4	22.5 22.1 22.6		21.7 20.6 18.2	19.0 21.7 20.0	20.0 20.5 20.4	21.3 20.5 21.7
Mean		**22.8**	**23.0**	**22.0**	**22.4**		**20.1**	**20.2**	**20.3**	**21.1**
SD		0.9	1.1	1.3	0.2		1.7	1.3	0.2	0.6
	CD59 (%)	16.3 17.1 16.2	16.5 16.8 17.0	15.7 16.8 16.2	15.9 16.8 16.4		17.1 16.1 16.9	24.7 23.5 24.4	23.7 21.9 22.0	22.7 22.3 21.7
Mean		**16.5**	**16.7**	**16.2**	**16.3**		**16.7**	**24.2**	**22.5**	**22.2**
SD		0.4	0.2	0.5	0.4		0.5	0.6	1.0	0.5
	CDC (%),	55.1 58.4 59.8	19.2 25.7 31.4	11.0 15.4 17.2	5.2 5.0 6.5		45.9 48.7 41.7	6.3 5.6 8.2	2.7 3.7 4.1	0.0 0.0 0.5
Mean		**57.7**	**25.4**	**14.5**	**5.5**		**45.4**	**6.7**	**3.5**	**0.1**
SD		2.4	6.1	3.1	0.8		3.5	1.3	0.7	0.2
	CDC (%), +mCRPs neutral.	40.0 41.1 43.0	29.9 34.1 36.1	22.3 22.4 19.6	7.6 8.1 13.5		37.0 35.9 42.8	10.0 7.8 8.5	3.7 3.2 4.4	0.2 0.4 o.2
Mean		**41.3**	**33.3**	**21.4**	**9.7**		**38.5**	**8.7**	**3.7**	**0.3**
SD		1.5	3.1	1.5	3.2		3.7	1.1	0.6	0.1
	fH binding (%)						11.6 12.4 15.8	17.8 29.4 30.0	39.2 39.8 43.4	35.0 38.8 33.2
Mean							**13.2**	**25.7**	**40.8**	**35.6**
SD							2.2	6.8	2.2	2.8
	iC3b (%)						11.6 9.0 11.8	21.9 18.4 17.8	15.4 18.9 21.3	24.7 23.7 23.8
Mean							**10.8**	**19.3**	**18.5**	**24.0**
SD							1.5	2.2	2.9	0.5

Chapter 4– Discussion

During the past three decades different treatment regimens for various kinds of tumors have been developed based on the availability of cancer-specific monoclonal antibodies. In general, the Ab-based therapy is considered effective but is not able not provide a complete cure for most of the cases. This led to the combined application of conventional chemo-and/or-radio-therapy with mAbs in a wide range of treatment regimens (Hurvitzet *et al.,* 2013; Jain and O'Brien, 2013).

The identification of biomarkers for tumor response to chemotherapy, radiotherapy or combined chemoradiation therapy represents a highly interesting research area with the aim to control treatment-associated toxicity and to provide optimal treatment strategies.

Many studies suggested that the cytotoxicity of chemotherapy, the CDC and the ADCC are efficiently regulated by shared groups of intracellular proteins (Reslan et al., 2009; Gancz & Fishelson, 2009). Accordingly, it was reasonable to expect in our current work a modified response to CDC and ADCC as a result of pre-treating cancer cells with various anti-cancer drugs. We therefore investigated the possible impact of clinically relevant chemotherapies on tumor cell susceptibilty to CDC and/or on the opsonisation of these cells with the C3 fragment iC3b. We also aimed to address the question if the alterations in complement effects are associated with a modified expression of membrane complement regulators. Since most of the previous studies were performed with long-term exposure of cancer cells to anti-cancer drugs, we wished to study if a modulatory effect could also occur after treating cancer cells for short intervals with selected clinically relevant drugs.

4.1. Breast cancer cell lines: impact of doxorubicin on mCRP expression, binding of fH, cell lysis and opsonisation with iC3

In soft tissue sarcomas (STS), response to chemotherapy has been associated with the expression of several complement genes (Zhang *et al.,* 2020). Subtypes of STS were relatively responsive to chemotherapies by expressing high levels of complement genes, a phenomenon considered to be of clinical significance (Zhang *et al.,* 2020).

We found a significant increase of CD55 and CD59 expression on BT474 cells upon treatment with doxorubicin for 24h. CD59 serves as biomarker for various tumors. Higher CD59 expression was associated with reduced survival in colorectal cancer patients (Watson *et al.,* 2006). An enhanced expression of CD46, CD55, and CD59, in a concentration-dependent manner, was also noticed in the cells treated for 48h.

In SKBR-3, doxorubicin significantly elevated CD46 and CD55 expression at 24h. At the same interval, alteration of CD59 expression was dependent on the drug concentration. At 48h, doxorubicin lead to elevated expression of CD46 on SKBR-3 in a concentration dependent manner but induced a significant decrease of the CD55 expression.

Higher expression of the mCRPs (CD46, CD55, and CD59) by cancer, compared to normal, cells has been widely reported (Seya *et al.,* 1994; Niehans *et al.,* 1996; Donin *et al.,* 2003, Fishelson and Kirschfink, 1999, 2019). Cancer cells exposed to various anti-cancer drugs showed variable levels of the mCRPs ranging from expression of higher CD59 and CD55 (Gelderman *et al.,* 2002) to lower levels of CD59 (Gorter *et al.,* 1996; Kuraya *et al.,* 1992) or CD55 (Maio *et al.,* 1998). This impact of anti-cancer drugs appears to be cell-type dependent: e.g. while daunomycin had no influence on CD59 expression in osteosarcoma cells (Bjørge and Matre, 1995), it induced over-expression of the regulator in ovarian carcinoma cells (A2780-MDR; Sedlak *et al.,* 1994).

In ovarian carcinoma, doxorubicin-resistant variants (OAW42-dox) expressing P-gp showed higher expression of CD59 and CD46 relative to parental cells (OAW42; Odening *at al.,* 2009). Upon treatment with tamoxifen, HER2-positive SKBR-3 and BT474 cells showed a reduction of CD55 but not of CD59 or CD46 on both RNA and protein levels (Mei Liu *et al.,* 2014).

In human oral carcinoma, multidrug-resistant cell variants (KB-V1) expressed a lower level of CD55 than parental cell line (KB-3-1; Bomstein *et al.,* 1997). Proteomic analysis of human plasma from breast cancer patients revealed modulation of the complement components by epirubicin and docetaxel as early as 24h following the initiation of treatment (Michlmayr *et al.,* 2010). This study suggested that certain C3 isoforms may have a potential as early predictive biomarkers for breast cancer response to epirubicin and docetaxel.

Both, complement fH and fHL-1 proteins bind to some tumors, including lung cancer cells (Ajona *et al.,* 2004). fH and fHL-1 proteins are soluble complement inhibitors which can bind to cell membranes through a complex process (Jozsi *et al.,*2004). Complement fH reacts with polyanionic surface proteins such as glycosamino-glycans which are often over-expressed by tumor cells (Ajona *et al.,* 2007; Fedarko *et al.,* 2000; Gasque *et al.,* 1992; Junnikkala *et al.,* 2002; Junnikkala *et al.,* 2000; Rodriguez de Cordoba *et al.,* 2004) through the C-terminal region and then induces inhibition of complement activation through its N-terminal region (Kopp *et al.,* 2012).

Our results demonstrate that treatment with doxorubicin for 48h significantly also enhance the binding of fH to BT474 cells, whereas this phenomenon was not observed in SKBR-3 cells. Besides the over-expression of the mCRPs, binding of fH to cancer cells is relevant to provide additional protection against unwanted complement activation (Jozsi *et al.,* 2004).

Activation of the complement system by tumor-targeting antibodies induces three main effector mechanisms: (a) opsonisation of target cells with C3 fragments like C3b and iC3b, (Perlmann *et al.,* 1981), (b) recruitment and activation of various immune cells by anaphylatoxins including C3a and C5a, (Markiewski *et al.,* 2008), and (c) formation of the membrane attack complex, C5b-9, which leads to tumor cell killing (Gelderman *et al.,* 2004).

We noticed that BT474 cells, exposed to doxorubicin for 24 and 48h, showed a significantly reduced sensitivity to the CDC. This can be attributed, at least in part, to the induction of CD55 and CD59 expression by doxorubicin at 24h and by over-expression of the three mCRPs at 48h. As treatment with doxorubicin for 48h also increased the binding of fH to BT474 cells, there is convincing evidence that doxorubicin induces an enhanced resistance to CDC even after short time exposure.

Also, SKBR-3 augmented the resistance to CDC after a 24h pretreatment with doxorubicin, which was also associated with over-expression of the three mCRPs. Binding of fH was higher in untreated SKBR-3 cells compared to BT474 cells and an additional increase of its binding to SKBR-3 cells was observed at 5μM doxorubicin. Also, here, the reduced susceptibility of these cells to complement lysis at 48h might be due to over-expression of both CD46 and CD55 and an enhanced binding level of fH.

In a previous work conducted by our research group, conversion of parental ovarian cells (OAW42) into a doxorubicin-resistant variant (OAW42-doxo) expressing P-gp was associated with a diminished sensitivity to CDC as a consequence of an over-expression of CD46 and CD55 (Odening *et al.,* 2009). This phenomenon was also reported for HL60 myeloid leukemia cells (Weisburg *et al.,* 1996), whereas doxorubicin-resistant human colon carcinoma cells were more sensitive to complement lysis than doxorubicin-sensitive cells (Gambacorti-Passerini *et al.,* 1988). Also, the multi-drug-resistant human oral carcinoma cell variants (KB-V1) exhibited a higher susceptibility to CDC compared to the parental cell variant (KB-3-1; Bomstein *et al.,* 1997). Anti-cancer drugs may not only induce tumor cell resistance to CDC but may also effectively inhibit NK-mediated cell killing with an obvious impact on immunotherapy (Markasz *et al.,* 2007).

In previous studies sensitivity of various tumor cells to CDC could be enhanced by post-transcriptional gene silencing and/or inhibition of mCRPs with neutralising antibodies (Jurianz *et al.,* 2001; Donin *et al.,* 2003; Zell *et al.,* 2007; Geis *et al.,* 2010; Mamidi *et al.*, 2013 & 2015). In our series of experiments, blocking mCRPs with specific antibodies induced a minor improvement in succeptibility of untreated cells to CDC and drug-treated cell lysis slightly augmented only with the higher drug concentrations. Neutralising Abs increased the lysis of untreated and of doxorubicin-treated SKBR-3 cells at 24, whereas at 48h, this effect was only seen in drug-treated cells at higher drug concentrations.

Activated C3b is converted into the inactive form iC3b by fI in the presence of fH as a cofactor. The iC3b opsonin serves as ligand for the CD11b/CD18 receptor on phagocytic cells (monocytes, macrophages, neutrophils, and dendiritic cells) to facilitate cell destruction by CDCC and by augmentation of ADCC (Mamidi *et al.,* 2015). In contrast to the poor susceptibility of drug-treated breast (BT474 and SKBR-3) cancer cells to CDC, we observed higher opsonisation levels with iC3b upon treatment with doxorubicin for 48h. This is in line with previous reports that knock down of CD46 and CD55 with siRNA significantly increased the opsonisation levels of tumor cells with iC3b (Geis *et al.,* 2010).

Collectively, our data suggest that combination of short-term application of doxorubicin with anti-cancer Abs supports tumor cell killing by both CDCC and ADCC. Further investigation is needed, to elucidate if Ab-induced mCRP inhibition also augments opsonisation with iC3b and subsequent impact on CDCC and ADCC.

4.2. Breast cancer cell lines: impact of taxol on mCRP expression, binding of fH, cell lysis and opsonisation with iC3

We also obsereved a higher expression of CD46 and CD59 in BT474 cells after 24h treatment with taxol. Only after 48h the expression of CD55 in BT474 cells significantly increased upon treatment, with the strongest effect at the highest drug concentration. In contrast, human ovarian carcinoma cells responded with a reduced expression of CD59 when treated with paclitaxel (John and George, 2012).

Earlier work of our group on human ovarian carcinoma cells indicated that taxol-resistant variants (OAW42-tax) expressing P-gp showed significantly higher expression levels of the mCRPs in the order: CD59 > CD46 > CD55 (Odening *et al.,* 2009). In our experiments, levels of mCRP expression on SKBR-3 cells were significantly increased after short-term treatment with taxol for 24h. Interestingly this effect was less pronounced after 48h exposure. Clinically, over-expression of CD55 and/or CD59 is considered as a marker for poor prognosis also reflecting the differentiation degrees of colorectal carcinoma (Durrant *et al.,* 2003; Watson *et al.,* 2006). In breast cancer, CD46 expression and the involvement of lymph nodes represent independent risk factors for disease-free survival. A less favorable diagnosis of this cancer was linked to the expression of this regulator (Maciejczyk *et al.,* 2011). Higher expression of CD55 in breast cancer was also associated with more resistance to apoptotic stimuli and to a higher growth rate (Ikeda *et al.,* 2008). This was suggested as an independent prognostic factor for the tumor recurrency (Ikeda *et al.,* 2008).

Binding of fH at 48h to taxol-treated BT474 cells was significantly higher than to untreated cells whereas binding of this regulator to taxol-treated SKBR-3 cells was not influenced. Although BT474 cells, treated with taxol for 24h, expressed significantly lower levels of CD46 and CD59 compared to untreated cells, these cells showed less sensitivity to complement lysis. This has also

been reported for tumor cells of different origin where reduced mCRP expression levels went along with a reduced susceptibility to CDC (Weisburg *et al.,* 1996; Bomstein and Fishelson 1997; Odening *et al.,* 2009). In contrast, exposure of BT474 to taxol for 48h resulted in a reduced CDC which could be explained with both, a slightly higher CD46 and CD55 expression and higher levels of fH binding.

Noteworthy, tumor cells can eliminate surface MAC complexes by shedding macrovesicles, allowing them to escape from complement-mediated lysis (Pilzer *et al.,* 2005; Pilzer and Fishelson, 2005). Exosomes confer resistance to treatment either by acting as a decoy for immunotherapies or by exporting drugs from cancer cells (Safaei *et al.,* 2005; Aung *et al.,* 2011). This suggests a possible additional mechanism behind the observed tumor cell resistance to CDC beyond the impact of complement regulators as investigated in our experiments.

CDC could be enhanced by inhibiting mCRPs by neutralising Abs. The antagonistic impact of both the membrane bound and soluble forms of fH on the sensitivity to complement attack has been widely documented (Schmidt *et al.,* 2008; Kopp *et al.,* 2012; Geller and Yan, 2019). Binding of fH to SKBR-3 cells was not influenced by taxol. However, these cells showed a significantly higher resistance to CDC at 24h due to a higher mCRPs expression, an association which was less pronounced at 48h. Here, mCRP neutralising Abs increased the sensitivity of breast cancer cells to CDC but only at the low drug concentrations.

Both, BT474 and SKBR-3, were better opsonised with iC3b after 48h taxol exposure. A higher opsonisation rate has already been previously reported after blocking the expression of CD46 and/or CD55 (Geis *et al.,* 2010).

4.3. Cancer cells of different origin: impact of bortezomib on mCRP expression, binding of fH, cell lysis and opsonisation with iC3b

Treatment of BT474 cells with bortezomib showed no influence on mCRPs expression at 24h but reduced the regulator levels at 48h. Exposure of SKBR3 cells to bortezomib for 24h elevated the mCRPs expression especially at the highest drug concentration. However, no impact for this drug on mCRP expression was noticed at 48h. Bortezomib-treated Raji cells for 24h showed a partial increase of CD46 and CD59 expression wereas CD55 expression was significantly increased. Although being out of the scope of the current study, in

various tumors several cytokines including IL-1α, IL-1β, IL-4, EGF, TNF-α, IFNγ, and Prostaglandin E2 regulate CD55 expression (Andoh *et al.,* 1996; Nasu *et al.,* 1998; Varsano *et al.,* 1998b; Takeuchi *et al.,* 2001; Wang and Dubois, 2006). Therefore, if the synthesis and secretion of some cytokines by the relevant cancer cells were influenced by pre-treatment with bortezomib needs further investigation. At 48h, mCRP expression was comparable on drug-treated and untreated cells. Expression of mCRP by cancer cells in response to various anti-cancer drugs was variable ranging from expression of higher CD59 and CD55 (Gelderman *et al.,* 2002) to lower levels of CD59 (Gorter *et al.,* 1996; Kuraya *et al.,* 1992) or CD55 (Maio *et al.,* 1998). This influence was also cell-type dependent (Bjørge and Matre, 1995; Sedlak *et al.,* 1994).

Higher levels of fH were bound to bortezomib-treated BT474 cells at 48h in particular at the highest drug concentration wereas binding of fH to drug-treated SKBR-3 cells was not influenced. In Raji cells, binding to fH to drug-treated cells was significantly higher than on untreated cells.

Our results showed a comparable sensitivity of untreated and bortezomib-treated BT474 cells to CDC at 24h. In presence of mCRP neutralising Abs, drug-treated cells had a higher susceptibilty to CDC, in particular at the highest concentration. This finding can also be explained by the lack of impact of bortezomib on the expression of mCRPs on BT474 cells at this time point. Upon blocking the regulatory activity of the mCRPs by neutralising Abs, a strong synergistic effect on tumor cell killing by CDC was achieved, in particular at the highest drug concentration.

BT474 cells treated with bortezomib for 48h showed a reduced susceptibility to CDC both in absence and presence of the mCRP neutralising Abs. Interestingly, although bortezomib-treated BT474 cells responded with a down-regulation of mCRP expression, binding of fH to drug-treated cells was increased which could explain the observed resistance to the CDC

Also, bortezomib-treated SKBR-3 cells for 24h were highly resistant to CDC, going along with a drug-induced elevation of mCRPs expression. At 48h drug-treated cells were as sensitive to CDC as untreated cells. Only after neutralising the mCRPs, sensitivity of these cells to CDC was enhanced. Although, bortezomib had no impact on the expression of the mCRPs, drug-treated cells bound high levels of fH which could account for the observed

enhanced resistance to CDC. Previous results indicated that apoptotic and/or necrotic actions induced by trastuzumab on HER-2 positive cells were synergistically enhanced upon treatment with bortezomib which was explained by targeting the NF-kappa B and p27 pathways by both drugs (Cardoso *et al.,* 2006).

Raji cells treated with bortezomib for 24h were highly resistant to CDC. This is going in line with the observed higher CD46, CD59 and CD55 expression induced by this drug at this interval. In various lymphoma cell lines, over-expression of the mCRPs was associated with a limited sensitivity to rituximab-mediated CDC (Golay *et al.,* 2000; Macor *et al.,* 2007; Horl *et al.,* 2013). In B-cell lymphoma, consumption of complement by exosomes might be implicated in protection against anti-CD20 antibodies (Aung *et al.,* 2011), although it has not been studied here, it could not be excluded as an additional factor for the observed resistance of Raji cells to CDC.

At 48h, bortezomib-treated cells showed the same sensitivity to CDC like that of untreated cells. An increase in susceptibility to CDC only occurred in presence of the mCRP neutralising Abs. Rituximab-treated B cell lymphoma with blocked activity of CD55 and CD59 were significantly more susceptible to CDC and the higher impact was associated with CD55 than CD59 (Golay *et al.,* 2000). Interestingly, expression of the mCRP by drug-treated and untreated Raji cells was comparable at this time point. However, binding to fH to drug-treated cells was significantly higher than to untreated cells which, like in SKBR-3, might be the cause behind the observed resistance of these cells to CDC.

BT474 cells treated with bortezomib were stronger opsonised with iC3b at the two highest used drug concentrations. This could be due to the obeserved reduction of CD46 and CD55 expression which confirms the previously reported data by our research group (Geis *et al.*, 2010).

Although exposure of SKBR-3 cells to bortezomib for 48h showed no impact on mCRP expression, these cells were opsonised with a higher level of iC3b and this is worth more investigations. Our results suggest that the combination of bortezomib and trastuzumab to treat HER-2 positive breast cancer may be a good choice to benefit from the synergistic effect of tumor cell killing by CDC, CDCC and ADCC.

The lack of impact of bortezomib on iC3b opsonisation of Raji cells goes along with a missing influence of this drug on mCRP expression.

4.4. Raji cells: impact of fludarabine on on mCRP expression, binding of fH, cell lysis and opsonisation with iC3

While expression of the mCRPs on both fludarabine-treated and untreated Raji cells was comparable after 24h, expression of CD46 in drug-treated cells was significantly reduced at 48h. A previous study revaled that activation of nuclear factor κB (NF-κB) is critical for CD46 expression (Ni Choileain *et al.,* 2017) which might reflect a possible interplay between fludarabine and the signaling cascade of NF-κB, an attractive link that needs to be addressed in the future. In contrast, expression of CD59 was significantly increased in drug-treated cells at the same time interval. CD59 has a biomarker value in tumors. High expression of CD59 was associated with decreased overall survival and progression-free survival in patients with diffuse large B cell lymphoma and adenocarcinomas of the prostate (Xu *et al.,* 2005; Song *et al.,* 2014). Patients with various refractory B-cell malignancies expressed significantly elevated levels of CD59 (Golay *et al.,* 2000; Treon *et al.,* 2001). In a follicular cell lymphoma cell line, expression of CD55 was down-regulated in response to fludarabine (Di Gaetano *et al.,* 2001).

At 24h, fludarabine-treated Raji cells were significantly less sensitive to CDC, although both, drug-treated and untreated cells expressed comparable levels of mCRPs. At 48h, drug-treated Raji cells were less sensitive to CDC compared to untreated cells. This could be explained by both the induction of a higher expression level of CD59 and stronger fH binding to the drug-treated cells. Previous reports showed synergistic impact of both fludarabine and rituximab on follicular cell lymphoma cytotoxicity which was linked to down-regulation of CD55 expression by the two agents (Di Gaetano *et al.,* 2001). Certainly, additional complement regulation mechanisms might be involved in the observed effect of fludarabine on CDC and this deserves further investigation. Fludarabine-treated Raji cells for 48h were highly opsonised with iC3b which could enhance the susceptibility of these cells to CDCC and ADCC.

From our point of view, the variable effect of mCRP-neutralising Abs on CDC of the studied cancer cells may be accredited to the following: (1) different expression mCRP levels among different tumor cell types, (2) differences

between the used in vitro cell culture system and - more relevant – (3) the conditions in vivo of the tumor microenvironment regarding both the type and the concentration of the released cytokines. Previous studies have shown that an inflammatory environment induces over-expression of mCRPs due to secretion of pro-inflammatory cytokines (Andoh *et al.,* 1996; Nasu *et al.,* 1998; Fang *et al.,* 2011).

Moreover, the sublytic insertion of MACs into the tumor cell membrane is associated with the activation of the PI3K/Akt signaling pathway, as seen by the inhibition of apoptosis in oligodendrocytes, and contribution to poor therapeutic responses (Song *et al.,* 2014). This highlights a potential involvement of this signaling pathway in the here whitnessed cells response to C5b-9.

4.5. Influence of different anti-cancer drugs on the secretion of soluble complement inhibitors by different cancer cell lines

Soluble complement regulatory proteins like C1-Inh, fI and fH are produced by many cancer cells and considered to contribute to complement regulation in the tumor microenvironment (Ziegler *et al.,* 2001). Secretion of C1-Inh and fI by the parental ovarian carcinoma cell line (OAW42) and taxol-resistant variant (OAW42-Tax) of these cells has been previously reported by our group (Odening *et al.,* 2009). Interfering with these soluble inhibitors conferred more susceptibility of ovarian carcinoma cells to CDC even at higher expression levels of the mCRPs (Unnikkala *et al.,* 2000).

In the current work secretion of soluble complement regulatory proteins (C1-Inh, fI and fH) into cell culture medium could not be detected by ELISA. Obviously, concentrations of these regulatory proteins were below the detection limit of our test system. We also cannot rule out that the selected cancer cells are inable to secrete any of these regulatory proteins under our experimental conditions. Since the major aspect of this study was to investigate the possible impact of anti-cancer drugs on tumor cell killing and/or opsonisation we did not put more efforts to this experimental line.

In conclusion, our results not only support previous findings but add more informations regarding the possible role of anti-cancer drugs to modulate the susceptibility of cancer cells to complement-mediated cytotoxicity. Although to a different extent, the drug-induced modulation of mCRP expression as well as higher fH binding levels in general augmented the resistance of cancer cells to

CDC. However, this may be counter- balanced by a better opsonisation of drug-treated cells with the opsonine iC3b which may potentially augment the destruction of the tumor by CDCC and ADCC.

4.6. Future outlook

To better estimate the influence of the applied anti-cancer drugs on the opsonisation of cancer cells with iC3b, further work is needed. This includes to analyse in more detail the possible role of immune effector cells like macrophages, NK-cells and neutrophils in the eradication of iC3b- opsonised tumor cells by CDCC and ADCC through their interaction with the complement receptor 3 (CR3, CD11b/CD18).

Further investigations are also needed to increase our understanding about the molecular mechanisms behind a stronger fH binding to drug-treated cancer cells and its impact on tumor cell opsonisation and CDC. Interfering with fH binding to drug-treated cells may be a strategy to increase eradication of solid tumor cells by CDC. This, however, requires a deeper knowledge on the relative potency of an improved CDC versus a stronger CDCC/ADCC upon successful opsonisation with iC3b.

Finally, both fH and iC3b fragments may be considerd as targets for CD8 positive T cells through bi-specific antibodies to provide effective cancer cell killing. This requires further evaluation in appropriate animal models bearing human cancer xenografts.

Summary

Although widely used in cancer therapy, chemotherapeutic drugs and anti-cancer antibodies are still unable to provide a desirable cure for all cancer patients. A major obstacle is the development of resistance mechanisms against chemotherapeutic drugs and immuotherapy. Most tumors are resistant to complement-mediated cytotoxicity (CDC), primarily due to over-expression of the membrane-associated regulatory proteins (mCRP) CD46, CD55 and CD59. This complement resistance has been shown to be modified (mostly augmented) not only by inflammatory cytokines and even sublytic complement and perforin, but also by various chemotherapeutic drugs in-vitro upon long-term treatment.

The present work aimed at analyzing the possible short-term impact of selected chemotherapeutic drugs on tumor cell lysis by CDC and/or opsonisation as well as the potential involvement of complement regulatory proteins.

A semi-kinetic approach was carried out in this study by choosing two different intervals (24h, 48h), applying different concentrations of the chemotherapeutic drugs doxorubicin, taxol, fludarabin and bortezomib on cancer cell lines of various histological origin (breast: BT474; SKBR-3; Lymphoma: Raji). Expression of mCRP, cancer cells opsonisation with iC3b and binding of the soluble complement regulator factor H to cancer cells were assessed by cytofluorometric analysis, cytotoxicity by ^{51}Cr-release assay and secretion of soluble inhibitors by ELISA.

The results indicate a close association between the modified expression of the mCRP and response of cancer cells to CDC. The strongest influence on expression was on CD55 and CD59. I could show for the first time that bortezimib inhibits mCRP expression on breast cancer cell lines leading to increased sensitivity to CDC. Although expression of membrane complement regulators was not enhanced in Raji cells upon treatment with bortezomib, a reduced sensitivity to CDC was still observed. Soluble complement inhibitors, such as C1-inhibitor, fH and fI potentially contributing to resistance of cancer cells to complement, were not detectable. However, I could demonstrate that drug-treated cells often efficiently bound higher levels of fH, in part in a dose

dependency manner, another possible protective mechanism against complement attack.

To improve the synergistic effect of the combined treatment regimens, I tried to abolish drug-induced complement resistance by blocking membrane regulators using specific neutralising antibodies. Neutralisation of the mCRP partially enhanced sensitivity of doxorubicin-treated BT474 cells to CDC but not of taxol–treated cells. In SKBR-3 cells treated with taxol for 48h, mCRP neutralisation could enhance their sensitivity to CDC, but was only partially able to improve sensitivity of bortezomib-treated and fludarabine-treated Raji cells to complement-mediated lysis. Interestingly, drug-treated cancer cells were often more efficiently opsonised with iC3b than untreated cells.

In conclusion, the here presented data demonstrate that even upon short-term exposure cancer cells develop an increased resistance to CDC. However, a more efficient opsonisation with iC3b could enhance their elimination through an augmented complement-mediated cellular cytotoxicity (CDCC) and antibody-dependent cellular cytotoxicity (ADCC).

Zusammenfassung

Trotz des zunehmenden Einsatzes von Chemotherapeutika, aber auch von therapeutischen Antikörpern ist eine Heilung von Tumorpatienten auch heute noch oftmals nicht möglich. Ein großes Hindernis stellt dabei die Resistenzentwicklung gegenüber Chemotherapeutika, aber auch gegenüber immuntherapeutischen Ansätzen dar. Die meisten Tumoren sind unempfindlich gegenüber einem Komplement-vermittelten cytotoxischen Angriff, was im Wesentlichen auf die Überexpression der Komplement-Membranregulatoren (mCRP) CD46, CD55 und CD59 zurückzuführen ist. Nicht nur inflammatorische Zytokine und selbst sub-lytisches Komplement und Perforin verändern (meist verstärken) diese Komplementresistenz, sondern auch verschiedene chemotherapeutische Medikamente in vitro nach Langzeitbehandlung der Tumorzellen.

Ziel der hier dargestellten Dissertation war es, den Einfluss einer Kurzzeitbehandlung ausgewählter Chemotherapeutika auf die Antiköper-induzierte, Komplement-vermittelte Opsonisierung und cytotoxische Zerstörung von Krebszellen zu untersuchen. In einem semikinetischen Ansatz mit 2 Inkubationszeiten (24 und 48 Stunden) wurden Zellen von Tumorzelllinien verschiedenen histologischen Ursprungs (Brusttumoren: BT474; SKBR-3; Lymphom: Raji) mit unterschiedlichen Konzentrationen der Chemotherapeutika Doxorubicin, Taxol, Fludarabin und Bortezomib behandelt.

Die Expression der mCRP, die Opsonisierung mit iC3b sowie die Bindung des Regulators Faktor H (fH) wurden mittels cytofluorometrischer Analyse, die zytotoxische Zerstörung mittels ^{51}Chrom- Freisetzungstest und die löslichen Komplementregulatoren (C1-Inhibitor, Faktor H und Faktor I) mittels ELISA quantifiziert.

Die Ergebnisse zeigen einen deutlichen Einfluss der mCRP-Expression auf die Empfindlichkeit der Tumorzellen gegenüber dem zytolytischen Komplement-Angriff. Die stärkste Wirkung übten die Chemotherapeutika auf die Regulatoren CD55 und CD59 aus. Ich konnte erstmalig zeigen, dass Bortezomib die Expression der Membranregulatoren auf Brusttumorzellen hemmt und dies zu einer erhöhten Sensitivität gegenüber der Komplement-vermittelten Zytotoxizität führt. Diese erhöhte Empfindlichkeit gegenüber

Bortezomib wurde auch bei Raji-Zellen beobachtet, obwohl dies nicht mit einer verminderten Expression der mCRP einherging. Eine Freisetzung der löslichen Regulatoren C1-Inhibitor, Faktor H und Faktor I aus unseren Tumorzellen konnte nicht nachgewiesen werden. Es gelang jedoch der Nachweis, dass, abhängig von der Dosis, Chemotherapeutika-behandelte Krebszellen Faktor H binden und damit eine mögliche weitere Steigerung der Komplementresistenz dieser Tumoren erreicht wird.

Um den synergistischen Effekt der Chemo- und Immuntherapie zu verbessern, versuchten wir die unter Einfluss der Chemotherpeutika hochexpremierten mCRP durch spezifische neutralisierende Antikörper zu blockieren. Diese Blockade führte zum Teil zu einer besseren zytotoxischen Zerstörung der mit Doxorubicin, jedoch nicht von mit Taxol behandelten BT474 Zellen. Bei den über 48 Stunden mit Taxol vorbehandelten SKBR-3 Zellen führte die Regulatorblockade zu einer erhöhten Empfänglichkeit gegenüber einem zytotoxischen Komplementangriff, was bei Bortezomib- und Fludarabin-behandelten Lymphomzellen jedoch nur teilweise gelang.

Interessanterweise wurden alle Chemotherapeutka-behandelten Tumorzellen, zum Teil dosisabhängig, besser durch Ablagerung von iC3b opsonisiert.

Zusammengefasst zeigen die hier präsentierten Daten zwar eine erhöhte Resistenz der Chemotherapeutik-behandete Tumorzellen gegenüber der Komplement-vermittelten Lyse auch nach Kurzzeitexposition, jedoch auch eine verstärkte Opsonisierung, was zu einer verbesserten Tumorzell-Elimination mittels Komplement-abhängiger zellulärer Zytotoxizität (CDCC) und Antikörper-abhängigen zellulären Zytotoxizität (ADCC) beitragen könnte.

Chapter 5- Bibliography

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Publications and Presentations

Thesis-related publications and posters

- 5th European Congress of Immunology (ECI) in Amsterdam from September 2-5, 2018.

  Poster:

  Mohamed H.A.H. Nasrh, Michael Kirschfink. Factor H (fH) binding and elevated expression levels of membrane bound complement regulators (mCRP) are key players of enhanced complement resistance of breast cancer cells upon treatment with paclitaxel and doxorubicin

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Research Experiences	
DAAD doctoral fellow (June 2016-present)	*''Impact of anti-cancer drugs on antibody-based complement-mediated tumor cell killing''* Institute of Immunology, University of Heidelberg, Germany **Advisor:** Prof. Dr. Michael Kirschfink
DAAD doctoral fellow (April 2015-May 2016)	*"Potential role of isg15-nonsense mutation in vasculitis of early childhood" in* Model systems for Infection and Immunity Research group, HZI, Braunschweig, Germany **Advisor:** Prof. Dr. Wirth Dagmar
Assistant researcher (2011-2014)	Assistant researcher in Immune- and Biomarkers for Infection Research Lab, National Research Center

	(NRC), Cairo, Egypt **Advisor:** Prof. Dr. Mahmoud Bahgat Riad
M.Sc. **(2008 –2011)**	*''Performance of an Egyptian vaccine prepared by reverse genetics from H5N1 Egyptian isolate against Avian Influenza Virus comparing with commercially available vaccines''* Virology Lab, National Research Center, (NRC) Cairo, Egypt **Advisor:** Prof. Dr. Mohamed Ahmed Ali
Microbiologist/ Specialist **(2006 –2009)**	Infectious diseases and Immunology Research Lab, National Research Center (NRC), Cairo, Egypt **Advisor:** Prof. Dr. Mahmoud Bahgat Riad

Honors & Awards

Recipient of DAAD (Deutscher Akademischer Austausch Dienst) GERLS fellowship program to pursue doctoral thesis in Germany 2014- present

Professional Association

Member, DGFI (2018)

Acknowledgement

A real and deep change of my scientific and social experiences has occurred in the last few years when I came to Germany to pursue my doctoral study and it would not have been possible without the support and guidance that I received on different levels.

I am grateful to the Deutscher Akademischer Austauschdienst (DAAD) for providing me the financial and research opportunities for my doctoral degree and stay in Germany.

Deep thanks for my supervisor **Prof. Dr. Michael Kirschfink** for providing me the opportunity to develop my doctoral thesis under his direct guidance at the Institute of Immunology, Medical Faculty, University of Heidelberg. He brought me for the first time to the complement world. Throughout my stay in Germany, **Prof. Kirschfink** did not only provide scientific support but also always took care of my social issues in an unusual friendly way. He has inspired me to become an independent researcher and helped me to realize the power of critical reasoning.

I would also like to acknowledge **Prof. Dr. Stefan Meuer**, Director of the Institute of Immunology for hosting me in the institute during my doctoral work.

I also would like to deeply thank **Prof. Dr. Mahmoud Bahgat,** my former M. Sc. Supervisor, who initially provided me a reference to win one of the very competitive DAAD long-term fellowship. It was he who introduced me to his former supervisor and current collaborator **Prof. Dr. Kirschfink.** During his visit to Heidelberg in 2018, Dr. Bahgat helped me to find out the best way to present my results graphically and statistically. He also critically read and corrected all sections of the work after the initial review by the referees.

My deep thanks go out to **Mrs. Chiara Rottaro** and to **Graduate Academy of Heidelberg University** for supporting me during the writing phaseof my thesis.

My thanks also go out to the support I received from my colleagues at the Institute of Immunology, Medical Faculty, University of Heidelberg, who were always helpful and shared their knowledge with me. A big appreciation goes to **Mrs. Renate Rutz** for making sure that everything went well in the lab on a day to day basis. I would also like to express my gratitude to **Mrs. Anja**

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In addition, I am truly indebted to **the National Research Center of Egypt (NRC)** for giving me the chance to study abroad and there were my first steps as a researcher. I am truly indebted to **Prof. Dr. Amany Sayed Maghraby** who not only helped me to figure out my scientific research career but also highly supported my scientific and social life.

I am grateful to **Dr. Ahmed Mostafa** and **Dr. Mahmoud Shehata** who were so friendly and supportive on different levels.

Last but not least, I would like to deeply thank the soul of my father, **Mr. Hassan A. Nasrh** which has been always with me, and my mother, **Mrs. Fatma Nasef**, for her endless love, support and patience. My parents have shaped me into the person I have become and achieved and for that I am truly indebted to them. I would also like to thank my siblings, **Mr. Ibrahim H. Nasrh** and **Mrs. Amal H. Nasrh**, who have always believed in me and were always there to cheer me up and motivate me. All of the love to my sons **Malek and Anas M. Hassan Nasrh**, they are being a special gift from God in my doctoral study time.

Eidesstattliche Versicherung

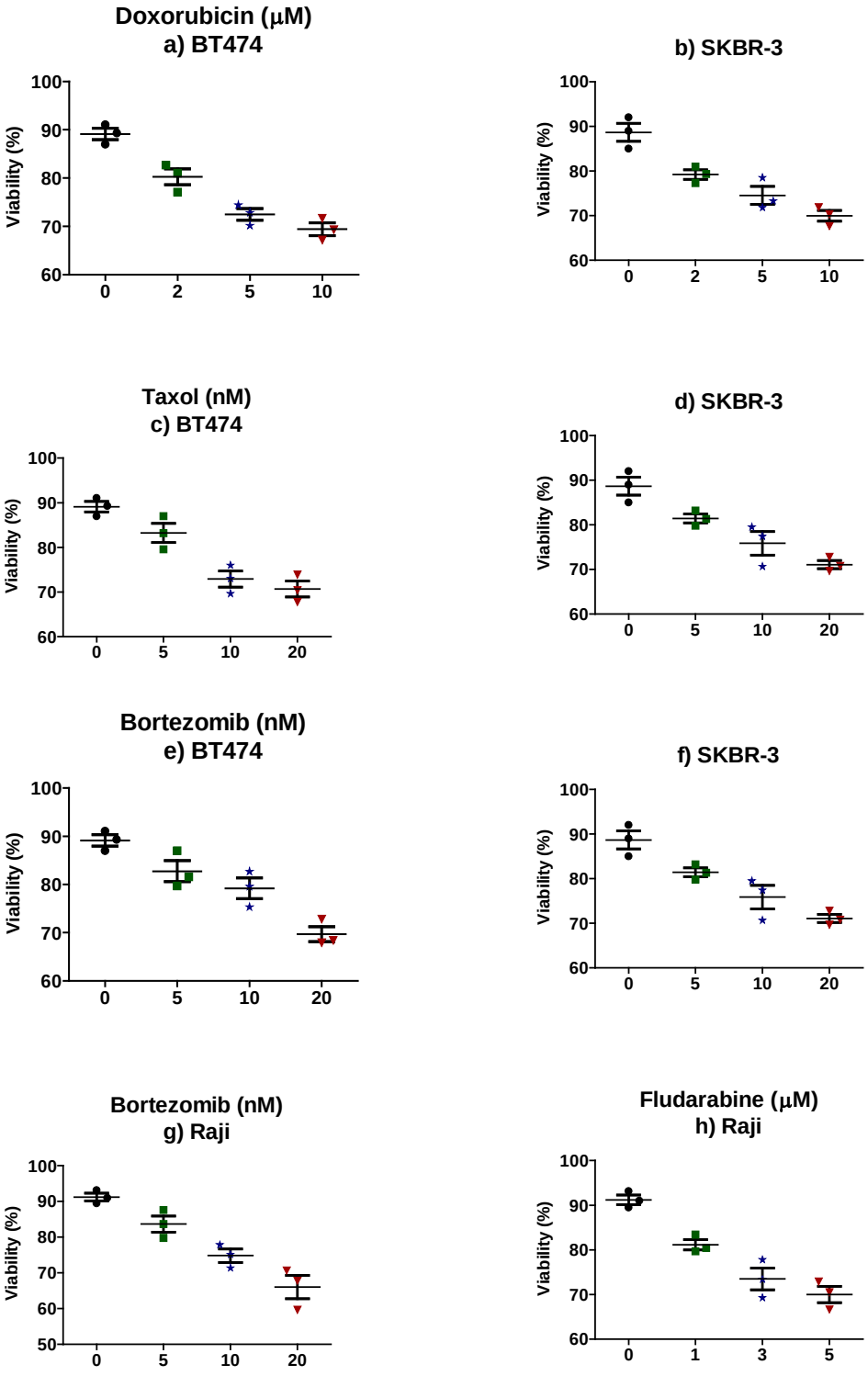
1. Bei der eingereichten Dissertation zu dem Thema
   “Impact of anti-cancer drugs on antibody-based complement-mediated tumor cell killing”
   handelt es sich um meine eigenständig erbrachte Leistung.
2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.
3. Die Arbeit oder Teile davon habe ich bislang nicht an einer Hochschule des In- oder Auslands als Bestandteil einer Prüfungs- oder Qualifikationsleistung vorgelegt.
4. Die Richtigkeit der vorstehenden Erklärungen bestätige ich.
5. Die Bedeutung der eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt. Ich versichere an Eides statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Heidelberg, den

Ort und Datum Unterschrift

Table 1: Viability of different cancer cell lines upon treatment with different chemotherapeutics for 48h

	Drugs															
	Doxorubicin (µM)				Taxol (nM)				Bortezomib (nM)				Fludarabine (µM)			
Cell line	0	2	5	10	0	5	10	20	0	5	10	20	0	1	3	5
BT474																
Viability (%)	91.1 89.3 87.0	82.7 77.1 81.0	74.4 72.8 70.2	71.7 67.2 69.3	91.1 89.3 87.0	83.2 87.0 79.6	73.1 76.0 69.7	70.4 73.9 67.8	91.1 89.3 87.0	87.0 81.6 79.7	82.7 79.6 75.3	72.8 68.4 67.9				
Mean	89.1	80.2	72.4	69.4	89.1	83.2	72.9	70.7	89.1	82.7	79.2	69.7				
SD	±2	±2.8	±2.1	±2.2	±2	±3.7	±3.1	±3.2	±2	±3.7	±3.7	±2.6				
SKBR-3																
Viability (%)	85.0 92.0 89.0	77.3 79.3 81.0	71.8 73.3 78.5	70.3 71.9 67.7	85.0 92.0 89.0	81.3 83.2 79.8	79.5 77.4 70.7	72.8 70.8 69.7	85.0 92.0 89.0	81.3 83.2 79.8	79.5 77.4 70.7	72.8 70.8 69.7				
Mean	88.6	79.1	74.5	69.4	88.6	81.4	75.8	71.1	88.6	75.4	68.8	63.7				
SD	±3.5	±1.8	±3.5	±2.1	±3.5	±1.7	±4.5	±1.5	±3.5	±3.9	±1.4	±3.5				
Raji																
Viability (%)									91.0 93.2 89.5	87.6 79.8 83.7	75.2 77.9 71.4	70.7 67.8 59.7	91.0 93.2 89.5	83.4 80.4 79.7	77.8 73.4 69.3	72.9 70.5 66.7
Mean									91.2	83.7	74.8	66.0	91.2	81.2	73.5	70.0
SD									±1.8	±3.9	±3.2	±5.7	±1.8	±1.9	±4.2	±3.1



Viability of different cancer cell lines upon treatment with different chemotherapeutics for 48h. (a&b) Viability of doxorubicin-treated BT474 & SKBR-3 cells. **(c&d**) Viability of taxol-treated BT474 & SKBR-3 cells. **(e, f & g)** Viability of bortezomib-treated BT474, SKBR-3 & Raji cells. **(h)** Viability of fludarabine-treated Raji cells. All cancer cells ($5x10^5$/well) were allowed to grow for 48h either without treatment or with indicated concentrations of each anti-cancer drug. Trypan blue exclusion assay was used to determine cell viability as described in Methods. Each symbol represents an independent experiment. Horizontal line: mean of three experiments; vertical line: SD.

The raw data (in cpm) presenting cell lysis (in %) by CDC of doxorubicin-treated BT474 cells at 24h

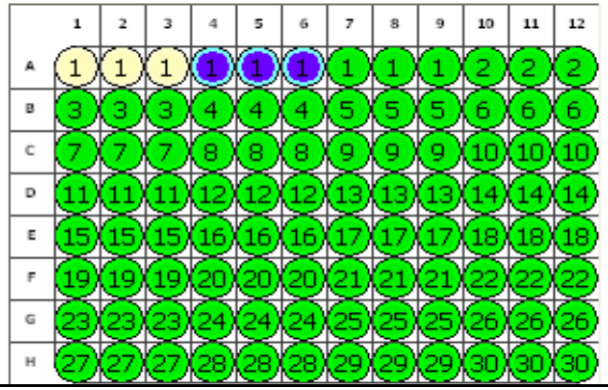
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1..1 and Pos Control1..1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 1: Raw data (in cpm) presenting cell lysis by CDC (in %) of doxorubicin-treated BT474 cells at 24h. The impact of pre-treatment of tumor cells with doxorubicin for 24h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneous release) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	728.42, 697.47, 913.43	0	1.1
Pos Control1	A4, A5, A6	1	11198.2, 12427.4, 10884.5	100	7.6
Unknown1	A7, A8, A9	1	552.38, 756.49, ~~966.69~~	4.8	4.9
Ut no Neut. A	A10, A11, A12	1	10368.2, 10140.1, 10393.4	88.8	1.3
Utno Neut. Ab	B1, B2, B3	1	10686.9, 8890.53, 8927.5	81.3	9.6
Ut no Neut. A	B4, B5, B6	1	8896.96, 9169.55, 6746.93	69.9	12.4
Ut Neut. Abs	B7, B8, B9	1	10379.8, 9197.56, 10024.2	84.7	5.7
Ut Neut. Abs	B10, B11, B12	1	9182.99, 9297.09, 9406.17	79.4	1
Ut Neut. Abs	C1, C2, C3	1	10144.2, 9167.47, 9085.57	81	5.5
2µM no Neut.	C4, C5, C6	1	4493.86, 4905.57, 4514.64	36	2.2
2µM no Neut.	C7, C8, C9	1	4565.83, 4311.1, 4518.84	34.4	1.3
2µM no Neut.	C10, C11, C12	1	4331.93, 4459.9, 4376.79	33.7	0.6
2µM Neut. Ab	D1, D2, D3	1	4657.4, 4657.58, 4993.52	37.2	1.8
2µM Neut. Ab	D4, D5, D6	1	4482.41, 4399.92, 4427.02	34.1	0.4
2µM Neut. Ab	D7, D8, D9	1	4360.29, 4527.8, 5113.52	36.3	3.7
5µM no neut.	D10, D11, D12	1	3451.82, 3862.92, 3449.5	26.2	2.2
5µM no neut.	E1, E2, E3	1	3688.88, 3413.89, 3843.49	26.8	2
5µM no neut.	E4, E5, E6	1	3707.16, 3350.12, 3427.86	25.3	1.8
5µM Neut. Ab	E7, E8, E9	1	4361.96, 4073.37, 4183.86	32	1.4
5µM Neut. Ab	E10, E11, E12	1	4094.37, 3754.39, 4244.11	30.3	2.3
5µM Neut. Ab	F1, F2, F3	1	4530.06, 3773.79, 4176.95	31.5	3.5
10µM no Neu	F4, F5, F6	1	4135.44, 3981.84, 3818.67	29.8	1.5
10µM no Neu	F7, F8, F9	1	4047.98, 3702.04, 3942.55	29.1	1.7
10µM no Neu	F10, F11, F12	1	3611.91, 3517.28, 3840.19	26.8	1.5
10µM Neut. A	G1, G2, G3	1	3602.63, 3477.39, 3762.95	26.4	1.3
10µM Neut. A	G4, G5, G6	1	3821.94, 3693.71, 4025.03	28.6	1.6
10µM Neut. A	G7, G8, G9	1	3911.97, 3771.05, 4106.07	29.4	1.6

The raw data (in cpm) presenting cell lysis by CDC (in %) of doxorubicin-treated BT474 cells at 48h.

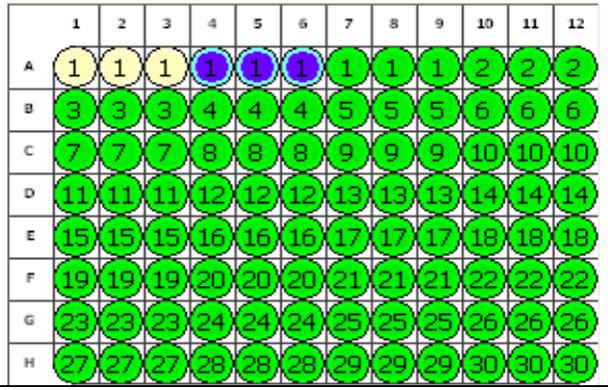
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1..1 and Pos Control1..1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 2: Raw data (in cpm) presenting cell lysis by CDC (in %) of doxorubicin-treated BT474 cells at 48h. The impact of pre-treatment of tumor cells with doxorubicin for 48h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneous release) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	342.69, 357.71, 402.37	0	1.2
Pos Control1	A4, A5, A6	1	3093.58, 3072.36, 3008.03	100	1.7
Unknown1	A7, A8, A9	1	552.38, 756.49, ~~966.69~~	4.8	4.9
Ut no Neut. A	A10, A11, A12	1	2854.18, 2932.65, 3012.72	95.4	2.9
Utno Neut. Ab	B1, B2, B3	1	2638.63, 2557.94, 2376.91	80.2	5
Ut no Neut. A	B4, B5, B6	1	2500.23, 3191.81, 3515.89	100.4	19.3
Ut Neut. Abs	B7, B8, B9	1	2439.36, 2250.59, 2459.27	74.9	4.3
Ut Neut. Abs	B10, B11, B12	1	2295.15, 2530.18, 2533.37	77.5	5.1
Ut Neut. Abs	C1, C2, C3	1	2760.4, 3006.94, 3129.39	96.6	7
2µM no Neut.	C4, C5, C6	1	1026.89, 1067.47, 1064.28	25.5	0.8
2µM no Neut.	C7, C8, C9	1	1001.13, 938.7, 948.94	22.1	1.2
2µM no Neut.	C10, C11, C12	1	983.32, 1008.12, 1375.27	28	8.2
2µM Neut. Ab	D1, D2, D3	1	1008.09, 1008.9, 1102.13	25	2
2µM Neut. Ab	D4, D5, D6	1	996.1, 924.5, 931.25	21.7	1.5
2µM Neut. Ab	D7, D8, D9	1	943.32, 899.63, 1174.52	23.7	5.5
5µM no neut.	D10, D11, D12	1	785.79, 839.93, 776.47	16.1	1.3
5µM no neut.	E1, E2, E3	1	901.34, 884.18, 868.04	19.2	0.6
5µM no neut.	E4, E5, E6	1	741.41, 883.86, 1467.41	16.5	3.7
5µM Neut. Ab	E7, E8, E9	1	832.21, 926.57, 834.95	18.5	2
5µM Neut. Ab	E10, E11, E12	1	803.59, 813.58, 852.92	16.9	1
5µM Neut. Ab	F1, F2, F3	1	854.18, 808, 939.98	18.6	2.5
10µM no Neu	F4, F5, F6	1	810.15, 966.53, 981.22	20.5	3.5
10µM no Neu	F7, F8, F9	1	849.26, 948.37, 799.91	18.5	2.8
10µM no Neu	F10, F11, F12	1	998.05, 765.07, 1129.67	22.2	6.9
10µM Neut. A	G1, G2, G3	1	952.55, 788.22, 937.86	19.5	3.4
10µM Neut. A	G4, G5, G6	1	904.79, 941.29, 1013.74	21.8	2.1
10µM Neut. A	G7, G8, G9	1	964.4, 985.58, ~~17.77~~	22.6	0.6

Raw data (in cpm) presenting cell lysis by CDC (in %) of doxorubicin-treated SKBR-3 cells at 24h

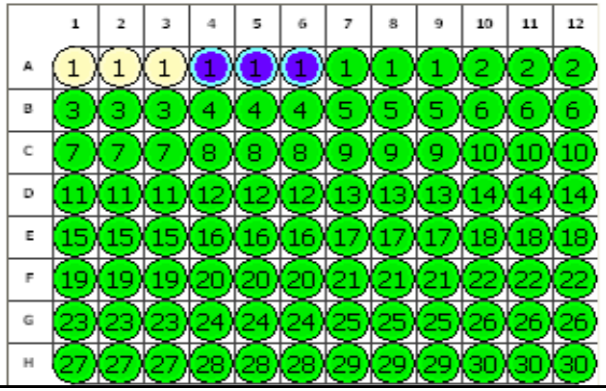
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1..1 and Pos Control1..1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 3: The raw data (in cpm) presenting cell lysis by CDC (in %) of doxorubicin-treated SKBR-3 cells at 24h. The impact of pre-treatment of tumor cells with doxorubicin for 24h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneous release) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	674.77, 583.64, 637.61	0	1.1
Pos Control1	A4, A5, A6	1	4590.16, 4998.2, 4867.02	100	5
Unknown1	A7, A8, A9	1	552.38, 756.49, ~~966.69~~	4.8	4.9
Ut	A10, A11, A12	1	3306.46, 3821.7, 3268.71	67.7	7.4
Ut	B1, B2, B3	1	3049.83, 2353, ~~677.04~~	49.4	11.8
Ut	B4, B5, B6	1	~~70.47~~, 39.82, 30.48	-14.3	0.2
Ut Neut. Abs	B7, B8, B9	1	3309.02, 3498.23, 3299.29	65.4	2.7
Ut Neut. Abs	B10, B11, B12	1	3257.25, 3507.84, 3483.66	66.5	3.3
Ut Neut. Abs	C1, C2, C3	1	2751.81, 2626, 2639.46	48.7	1.7
2µM	C4, C5, C6	1	1416.43, 1487.06, 1457.44	19.6	0.8
2µM	C7, C8, C9	1	1513.49, 1535.97, 1500.42	21.1	0.4
2µM	C10, C11, C12	1	1512.04, 1558.06, 1354.91	20.1	2.5
2µM Neut. Ab	D1, D2, D3	1	2149.32, 1985.57, 2163.87	35.1	2.4
2µM Neut. Ab	D4, D5, D6	1	2041.3, 1920.27, 1899.11	31.6	1.8
2µM Neut. Ab	D7, D8, D9	1	1917.17, 1892.49, 1914.48	30.5	0.3
5µM	D10, D11, D12	1	2369.65, 2441.39, 2322.57	41.7	1.4
5µM	E1, E2, E3	1	2462.14, 2241.21, 2256.34	40.3	2.9
5µM	E4, E5, E6	1	2257.34, 2163.7, 2048.9	36.4	2.5
5µM Neut. Ab	E7, E8, E9	1	1840.63, 1803.22, 1842.95	28.6	0.5
5µM Neut. Ab	E10, E11, E12	1	1836.92, 1953.26, 1835.16	29.7	1.6
5µM Neut. Ab	F1, F2, F3	1	2610.17, 2618.87, 2112.35	43.4	6.9
10µM	F4, F5, F6	1	2378, 2280.36, 2043.09	38.3	4.1
10µM	F7, F8, F9	1	2156.1, 2189.29, 2282.9	37.7	1.6
10µM	F10, F11, F12	1	2164.94, 2064.28, 2125.42	35.5	1.2
10µM Neut. A	G1, G2, G3	1	2139.28, 1997.19, 1916.93	33.1	2.7
10µM Neut. A	G4, G5, G6	1	2000.64, 2035.9, 2120.56	33.9	1.5
10µM Neut. A	G7, G8, G9	1	2196.53, 2260, 2237.97	38.2	0.8

Raw data (in cpm) presenting cell lysis by CDC (in %) of doxorubicin-treated SKBR-3 cells at 48h

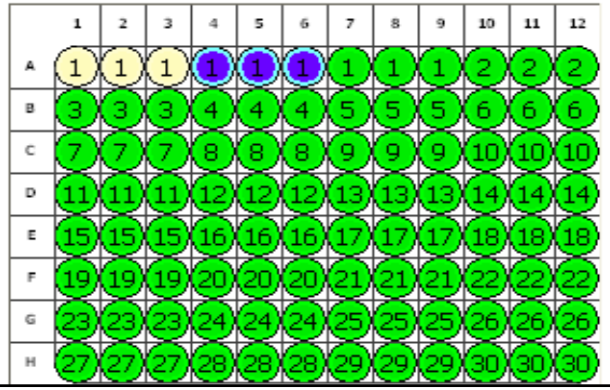
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1..1 and Pos Control1..1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 4: The raw data (in cpm) presenting cell lysis by CDC (in %) of doxorubicin-treated SKBR-3 cells at 48h. The impact of pre-treatment of tumor cells with doyorubicin for 48h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneous release) / (maximum release – spontaneous release)) × 100**.**

Raw data (in cpm) presenting cell lysis by the CDC (in %) of BT474 cells treated with taxol for 24h

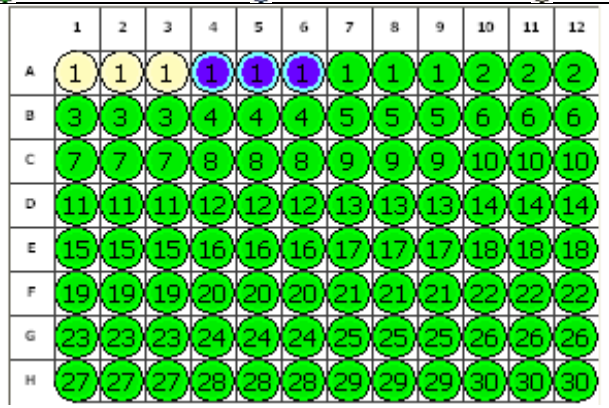
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 5: The raw data (in cpm) and the cell lysis by CDC (in %) of taxol-treated BT474 cells at 24h. The impact of pre-treatment of tumor cells with taxol for 24h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	363.14, 314.52, 331.66	0	0.5
Pos Control1	A4, A5, A6	1	5556.08, 5571.3, 5419.99	100	1.6
Unknown1	A7, A8, A9	1	1526.34, 1577.93, 1445.73	22.8	1.3
Ut -/- Neutr.A	A10, A11, A12	1	4173.54, 4544.37, 4556.91	78.9	4.2
Ut -/- Neutr.A	B1, B2, B3	1	4016.51, 3720.6, 3822.44	67.9	2.9
Ut -/- Neutr.A	B4, B5, B6	1	3903.03, 3752.7, 4417.9	71.2	6.7
Ut +/+ Neutr.	B7, B8, B9	1	2833.52, 3025.09, 3231.88	52	3.8
Ut +/+ Neutr.	B10, B11, B12	1	3177.11, 2981.02, 3031.71	52.6	2
Ut +/+ Neutr.	C1, C2, C3	1	3446.2, 3333.36, 3632.27	60.5	2.9
5nM -/- Neutr	C4, C5, C6	1	3061.28, 3974.07, 3504.91	61.3	8.8
5nM -/- Neutr	C7, C8, C9	1	3567.16, 3330.39, 3497.42	60.4	2.3
5nM -/- Neutr	C10, C11, C12	1	3580.44, 4174.77, 3313.59	64.7	8.5
5nM +/+Neut	D1, D2, D3	1	2809.44, 3589.49, 2862.62	53.1	8.4
5nM +/+Neut	D4, D5, D6	1	2770.54, 2970.13, 2925.71	49.3	2
5nM +/+Neut	D7, D8, D9	1	3188.41, 3412.65, 3599.7	59.2	4
10nM -/- Ney	D10, D11, D12	1	3351.82, 3474, 3819.07	62	4.7
10nM -/- Neu	E1, E2, E3	1	5648.31, 5664.44, 5306.65	100.5	3.9
10nM -/- Neu	E4, E5, E6	1	3564.54, 3747.65, 3560.62	63.5	2.1
10nM +/+Neu	E7, E8, E9	1	2633.37, 2817.6, 2819.34	46.7	2.1
10nM +/+Neu	E10, E11, E12	1	3180.75, 2707.98, 3021.9	50.9	4.6
10nM +/+Neu	F1, F2, F3	1	4724.91, 4425.34, 4764.99	83.1	3.6
20nM -/-Neut	F4, F5, F6	1	3388.3, 4079.43, 3820	66.1	6.7
20nM -/-Neut	F7, F8, F9	1	4004.56, 3933.77, 3982.15	70.2	0.7
20nM -/-Neut	F10, F11, F12	1	3974.41, 3970.13, 3939.02	70	0.4
20nM +/+Neu	G1, G2, G3	1	3022.79, 2627.09, 2827.48	48.1	3.8
20nM +/+Neu	G4, G5, G6	1	2751.8, 3021.94, 4250.21	58	15.4
20nM +/+ Ne	G7, G8, G9	1	4456.75, 4271.34, 4523.41	78.8	2.5

Raw data (in cpm) presenting cell lysis by CDC (in %) of taxol-treated BT474 cells at 48h

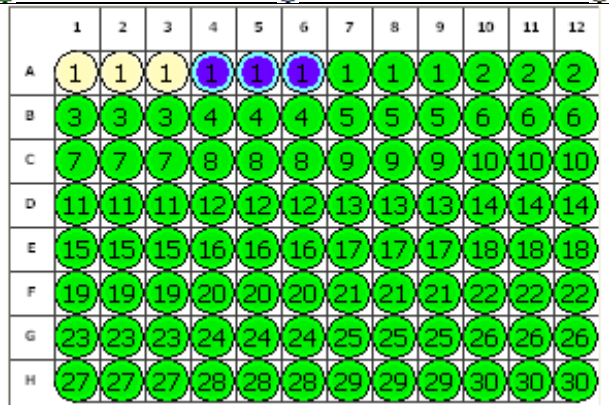
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 6: The raw data (in cpm) and the cell lysis by CDC (in %) of taxol-treated BT474 cells at 48h. The impact of pre-treatment of tumor cells with taxol for 48h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	373.48, 361.97, 408.83	0	0.5
Pos Control1	A4, A5, A6	1	5641.64, 5732.14, 5857.01	100	2
Unknown1	A7, A8, A9	1	3958.08, 3576.8, 4303.79	66.5	6.8
Ut -/- Neutr.A	A10, A11, A12	1	4675.06, 5025.99, 5017.76	84.4	3.7
Ut -/- Neutr.A	B1, B2, B3	1	5393.56, 4669.21, 4861.55	85.7	7
Ut -/- Neutr.A	B4, B5, B6	1	4655.94, 4917.36, 4735.18	81.8	2.5
Ut +/+ Neutr.	B7, B8, B9	1	2841.26, 3077.92, 2938.1	47.9	2.2
Ut +/+ Neutr.	B10, B11, B12	1	2773.44, 2917.72, 2753.66	45.4	1.7
Ut +/+ Neutr.	C1, C2, C3	1	2926.97, 3053.52, 3092.87	49.3	1.6
5nM -/- Neutr	C4, C5, C6	1	3566.55, 3944, 4191.55	65.6	5.9
5nM -/- Neutr	C7, C8, C9	1	4026.35, 3662.47, 3764.94	64.1	3.5
5nM -/- Neutr	C10, C11, C12	1	3565.45, 4221.27, 4909.23	71.8	12.5
5nM +/+Neut	D1, D2, D3	1	3179.44, 3329.11, 3652.04	56	4.5
5nM +/+Neut	D4, D5, D6	1	3003.91, 3459.47, 3203.48	53	4.3
5nM +/+Neut	D7, D8, D9	1	3640.61, 3300.72, 3425.34	57.3	3.2
10nM -/- Ney	D10, D11, D12	1	4332.49, 4806.08, 4681.32	78.8	4.6
10nM -/- Neu	E1, E2, E3	1	4375.24, 4113.71, 4563.76	74	4.2
10nM -/- Neu	E4, E5, E6	1	4128.77, 4264.36, 4551.32	73.4	4
10nM +/+Neu	E7, E8, E9	1	3969.12, 4455.57, 4688.05	74.4	6.8
10nM +/+Neu	E10, E11, E12	1	4036.43, 4229, 4037.14	69.4	2.1
10nM +/+Neu	F1, F2, F3	1	4377.12, 3941.16, 4510.38	72.6	5.6
20nM -/-Neut	F4, F5, F6	1	2918.98, 3255, 3123.75	50.7	3.2
20nM -/-Neut	F7, F8, F9	1	3184.85, 2874.49, 2945.15	48.9	3
20nM -/-Neut	F10, F11, F12	1	2880.43, 2973.23, ~~6731.3~~	47.5	1.2
20nM +/+Neu	G1, G2, G3	1	1827.49, 2281.6, 2590.71	34.5	7.2
20nM +/+Neu	G4, G5, G6	1	2715.42, 2061.88, 2261.83	36.6	6.2
20nM +/+ Ne	G7, G8, G9	1	2621.31, 2841.16, 2977.19	45.4	3.3

Raw data (in cpm) presenting cell lysis by CDC (in %) of taxol-treated SKBR3 cells at 24h

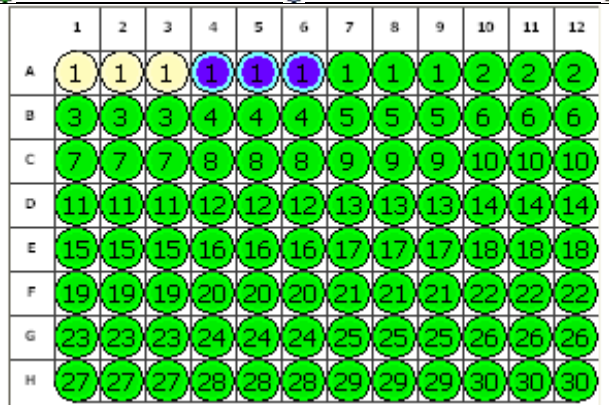
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 7: The raw data (in cpm) presenting cell lysis by CDC (in %) of taxol-treated SKBR-3 cells at 24h. The impact of pre-treatment of tumor cells with taxol for 24h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	404.19, 401.9, 425.79	0	0.4
Pos Control1	A4, A5, A6	1	3712.96, 3868.85, 4807.8	100	15.9
Unknown1	A7, A8, A9	1	~~571.36~~, 890.02, 1031.97	14.8	2.7
Ut -/- Neutr.A	A10, A11, A12	1	3463.17, 3370.17, 3218.45	79	3.3
Ut -/- Neutr.A	B1, B2, B3	1	3544.16, 3208.08, 3268.09	78.8	4.8
Ut -/- Neutr.A	B4, B5, B6	1	3493.23, 3772.45, 3678.41	87	3.8
Ut +/+ Neutr.	B7, B8, B9	1	2392.89, 2634.09, 2996.43	60.9	8.2
Ut +/+ Neutr.	B10, B11, B12	1	2555.13, 2580.35, 2515.08	57.5	0.9
Ut +/+ Neutr.	C1, C2, C3	1	2579.55, 2428.77, 3076.97	61.4	9.1
5nM -/- Neutr	C4, C5, C6	1	3269.81, 3643.79, 3155.63	79.2	6.9
5nM -/- Neutr	C7, C8, C9	1	3188.61, 3038.28, 3275.11	74.1	3.2
5nM -/- Neutr	C10, C11, C12	1	3511.27, 3776.38, 3981.97	90	6.3
5nM +/+Neut	D1, D2, D3	1	3006.78, 3203.24, 3145.12	72.8	2.7
5nM +/+Neut	D4, D5, D6	1	3079.27, 2939.27, 3011.48	69.9	1.9
5nM +/+Neut	D7, D8, D9	1	3149.08, 3062.48, 3404.61	75.1	4.8
10nM -/- Ney	D10, D11, D12	1	2648.44, 2657.55, 2704.55	60.8	0.8
10nM -/- Neu	E1, E2, E3	1	2671.35, 2493.53, 2873.15	61	5.1
10nM -/- Neu	E4, E5, E6	1	2718.04, 3010.18, 3738.89	73.8	14.1
10nM +/+Neu	E7, E8, E9	1	2074.33, 1952.82, 2268.97	45.4	4.3
10nM +/+Neu	E10, E11, E12	1	2126.1, 2393.08, 2288.41	50	3.6
10nM +/+Neu	F1, F2, F3	1	2494.05, 2588.2, 2700.41	58.7	2.8
20nM -/-Neut	F4, F5, F6	1	1981.83, 1967.21, 2059.99	42.8	1.3
20nM -/-Neut	F7, F8, F9	1	1947.4, 1673.61, 1831.72	37.8	3.7
20nM -/-Neut	F10, F11, F12	1	1916.61, 1729.18, 1699.37	36.9	3.2
20nM +/+Neu	G1, G2, G3	1	1942.91, 2105.56, 1958.16	42.8	2.4
20nM +/+Neu	G4, G5, G6	1	2050.38, 2084.31, 1934.84	43.4	2.1
20nM +/+ Ne	G7, G8, G9	1	1828.33, 1904.71, 1703.08	37.7	2.7

Raw data (in cpm) presenting cell lysis by CDC (in %) of taxol-treated SKBR3 cells at 48h

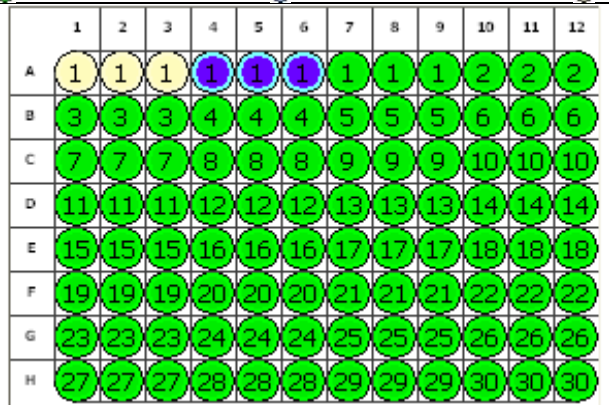
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 8: The raw data (in cpm) and the cell lysis by CDC (in %) of taxol-treated SKBR-3 cells at 48h. The impact of pre-treatment of tumor cells with taxol for 48h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	444.13, 391.78, 406.45	0	0.7
Pos Control1	A4, A5, A6	1	4681.46, 4531.37, 4397.8	100	3.4
Unknown1	A7, A8, A9	1	2032.52, 2416.43, 1677.23	39.5	9
Ut -/- Neutr.A	A10, A11, A12	1	3036.66, 3230.41, 3528.2	69.2	6
Ut -/- Neutr.A	B1, B2, B3	1	3439.95, 3264.1, 3224.42	70.2	2.8
Ut -/- Neutr.A	B4, B5, B6	1	3140.07, 3245.25, 3314.45	68.4	2.1
Ut +/+ Neutr.	B7, B8, B9	1	~~1936.28~~, 3484.34, 3012.15	68.7	8.1
Ut +/+ Neutr.	B10, B11, B12	1	2285.07, 2910.44, 2737.54	54.1	7.8
Ut +/+ Neutr.	C1, C2, C3	1	2841.56, 2973.66, 2617.47	58.1	4.4
5nM -/- Neutr	C4, C5, C6	1	2530.71, 2641.54, 2379.8	51	3.2
5nM -/- Neutr	C7, C8, C9	1	2395.55, 2435.17, 2428.57	48.6	0.5
5nM -/- Neutr	C10, C11, C12	1	2415.2, 2400.89, 2498	49.1	1.3
5nM +/+Neut	D1, D2, D3	1	3154.18, 3022.68, 3346.85	67	4
5nM +/+Neut	D4, D5, D6	1	2908.73, 3155.44, 2820.28	61.8	4.2
5nM +/+Neut	D7, D8, D9	1	3250.68, 3281.95, 3635.11	72.2	5.2
10nM -/- Ney	D10, D11, D12	1	2329.4, 2484.45, 2541.54	49.4	2.7
10nM -/- Neu	E1, E2, E3	1	2437.03, 2131.04, 2287.56	45.4	3.7
10nM -/- Neu	E4, E5, E6	1	1972.01, 2318.33, 2959.52	48.6	12.2
10nM +/+Neu	E7, E8, E9	1	2667.44, 2623.76, 2693.9	54.5	0.9
10nM +/+Neu	E10, E11, E12	1	2556.61, 2547.1, 2583.71	52.1	0.5
10nM +/+Neu	F1, F2, F3	1	2791.75, 2601.58, 2678.34	55.2	2.3
20nM -/-Neut	F4, F5, F6	1	506.45, 581.57, 530.81	3	0.9
20nM -/-Neut	F7, F8, F9	1	596.79, 555.69, 551.78	3.7	0.6
20nM -/-Neut	F10, F11, F12	1	576, 534.99, 606.66	3.8	0.9
20nM +/+Neu	G1, G2, G3	1	676.22, 797.41, 870.49	8.9	2.4
20nM +/+Neu	G4, G5, G6	1	526.88, 551.15, 541.79	3.1	0.3
20nM +/+ Ne	G7, G8, G9	1	601.97, 540.69, 554.62	3.7	0.8

Raw data (in cpm) presenting cell lysis by CDC (in %) of bortezomib-treated BT474 cells at 24h

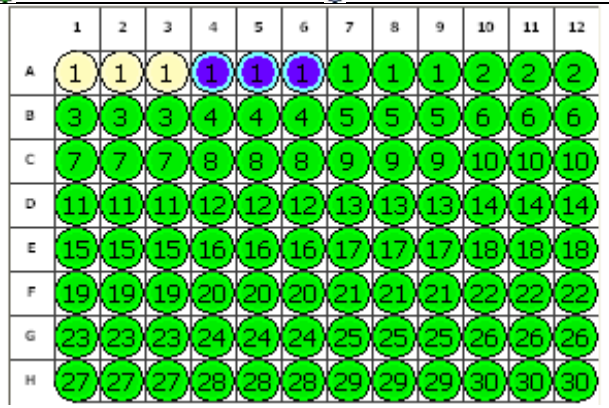
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 9: The raw data (in cpm) and the cell lysis by CDC (in %) of bortezomib-treated BT474 cells at 24h. The impact of pre-treatment of tumor cells with bortezomib for 24h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	677.18, 702.81, ~~1144.48~~	0	0.2
Pos Control1	A4, A5, A6	1	11890.3, 12275.2, 11479.4	100	3.6
Unknown1	A7, A8, A9	1	5895.37, 4079.1, 5204.62	39	8.2
Ut -/- Neutr.A	A10, A11, A12	1	8613.14, 9378.54, 8833.4	73.7	3.5
Ut -/- Neutr.A	B1, B2, B3	1	9522.84, 8108.82, 8323.98	71.1	6.8
Ut -/- Neutr.A	B4, B5, B6	1	8284.94, 8562.63, 8110.52	68.2	2
Ut +/+ Neutr.	B7, B8, B9	1	8933.98, 9772.39, 9550.13	78	3.9
Ut +/+ Neutr.	B10, B11, B12	1	8845.97, 9022.46, 8118.91	71.2	4.3
Ut +/+ Neutr.	C1, C2, C3	1	8948.62, 8367.13, 9869.03	74.8	6.8
5nM -/- Neutr	C4, C5, C6	1	8196.97, 7968.2, 8104.97	66.1	1
5nM -/- Neutr	C7, C8, C9	1	9095.92, 8648.4, 8989.43	73.5	2.1
5nM -/- Neutr	C10, C11, C12	1	6952.12, ~~2719.11~~, 7023.69	56.3	0.5
5nM +/+Neut	D1, D2, D3	1	9142.38, 9450.28, 9334.22	77	1.4
5nM +/+Neut	D4, D5, D6	1	8733.33, 8280.7, 8574.64	70	2.1
5nM +/+Neut	D7, D8, D9	1	8612.16, 8430.79, 9035.37	71.5	2.8
10nM -/- Ney	D10, D11, D12	1	6996.39, 8085.34, 8810.79	65	8.2
10nM -/- Neu	E1, E2, E3	1	9718.46, 9540.65, 11080.5	84.2	7.5
10nM -/- Neu	E4, E5, E6	1	9327.39, 9676.79, 9380.16	78.4	1.7
10nM +/+Neu	E7, E8, E9	1	10148.2, 9612.37, 9933.51	82.3	2.4
10nM +/+Neu	E10, E11, E12	1	9668.78, 8820.97, 9104.18	76	3.9
10nM +/+Neu	F1, F2, F3	1	6601.49, 6687.66, 8622.63	59.1	10.2
20nM -/-Neut	F4, F5, F6	1	9533.42, 10552.7, 9717.1	82.6	4.9
20nM -/-Neut	F7, F8, F9	1	9514.67, 9366.69, 9313.68	77.8	0.9
20nM -/-Neut	F10, F11, F12	1	9272.51, 9646.59, 9384.51	78.1	1.7
20nM +/+Neu	G1, G2, G3	1	10288, 10629.5, 11368.6	90	4.9
20nM +/+Neu	G4, G5, G6	1	10947.8, 10782.1, 10097.1	88.6	4
20nM +/+ Ne	G7, G8, G9	1	11155.8, 10397.4, 10755.8	90.1	3.4

Raw data (in cpm) presenting cell lysis by CDC (in %) of bortezomib-treated BT474 cells at 48h

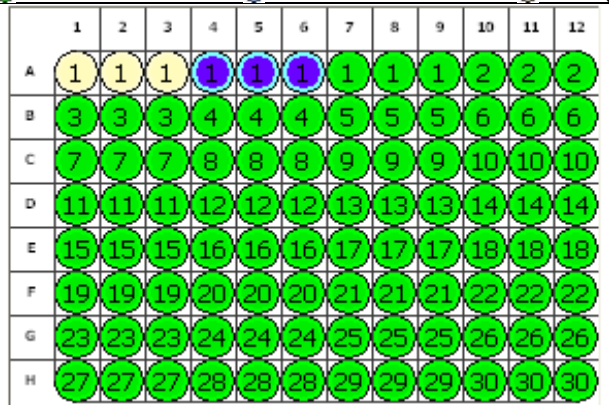
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 10: The raw data (in cpm) and the cell lysis by CDC (in %) of bortezomib-treated BT474 cells at 48h. The impact of pre-treatment of tumor cells with bortezomib for 48h on CDC either in absence or in presence ofmCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	385.52, 416.49, 424.83	0	0.6
Pos Control1	A4, A5, A6	1	3719.68, 4503.21, 4285.5	100	10.8
Unknown1	A7, A8, A9	1	3877.92, 3819.39, 3925.19	92.1	1.4
Ut -/- Neutr.A	A10, A11, A12	1	3704.83, 3946.88, 4020.1	92.6	4.4
Ut -/- Neutr.A	B1, B2, B3	1	4087.22, 3711.66, 3989.99	93.6	5.2
Ut -/- Neutr.A	B4, B5, B6	1	3324.83, 4341.01, 5256.48	103.7	25.7
Ut +/+ Neutr.	B7, B8, B9	1	3761.78, 3664.69, 3684.98	87.6	1.4
Ut +/+ Neutr.	B10, B11, B12	1	3268.79, 3511.6, 3460.39	79.9	3.4
Ut +/+ Neutr.	C1, C2, C3	1	3998.67, 3896.6, ~~6199.59~~	94.1	1.9
5nM -/- Neutr	C4, C5, C6	1	2809.66, 3167.24, 2625.33	65.4	7.3
5nM -/- Neutr	C7, C8, C9	1	3172.9, 3014.38, 2819.43	69	4.7
5nM -/- Neutr	C10, C11, C12	1	2561.62, 3001.84, ~~4758.87~~	63.1	8.3
5nM +/+Neut	D1, D2, D3	1	2903.08, 3275.66, 3121.14	71.6	5
5nM +/+Neut	D4, D5, D6	1	3056.43, 3191.59, 3220.46	73.1	2.3
5nM +/+Neut	D7, D8, D9	1	2887.89, 2898.23, 2899	66.1	0.2
10nM -/- Ney	D10, D11, D12	1	3077.74, 2953.47, 3057.76	69.7	1.8
10nM -/- Neu	E1, E2, E3	1	3042.81, 2840.56, 3103.89	68.8	3.7
10nM -/- Neu	E4, E5, E6	1	2802.11, 3110.89, 4125.43	78.1	18.4
10nM +/+Neu	E7, E8, E9	1	2163.92, 2719.06, 2615.92	55.6	7.9
10nM +/+Neu	E10, E11, E12	1	2393.66, 2628.4, 2495.19	55.8	3.1
10nM +/+Neu	F1, F2, F3	1	~~2483.46~~, 4505.9, 4315.6	106.4	3.6
20nM -/-Neut	F4, F5, F6	1	2815.99, 2964.46, 2828.06	65.4	2.2
20nM -/-Neut	F7, F8, F9	1	3162.3, 2890.74, 2842.21	68	4.6
20nM -/-Neut	F10, F11, F12	1	2788.35, 3029.66, 3761.05	74	13.5
20nM +/+Neu	G1, G2, G3	1	2507.22, 2922.25, 2982.4	63.7	6.9
20nM +/+Neu	G4, G5, G6	1	2837.06, 2862.26, 2723.45	63.8	2
20nM +/+ Ne	G7, G8, G9	1	2818.6, 2739.49, ~~30.08~~	63	1.5

Raw data (in cpm) presenting cell lysis by CDC (in %) of bortezomib-treated Raji cells at 24h

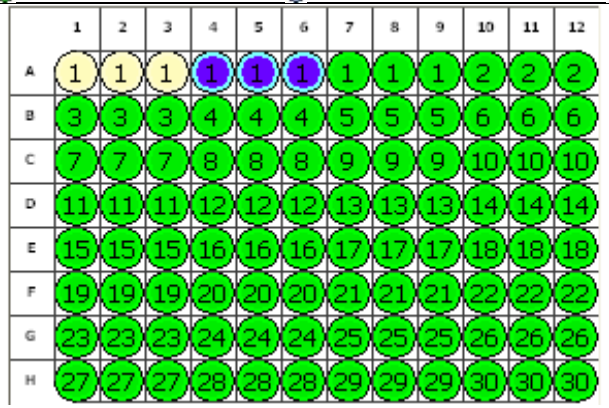
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 11: The raw data (in cpm) and the cell lysis by CDC (in %) of bortezomib-treated Raji cells at 24h. The impact of pre-treatment of tumor cells with bortezomib for 24h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	401.9, 375.81, 376.93	0	0.3
Pos Control1	A4, A5, A6	1	5381.07, 4942.06, 7607.89	100	25.6
Unknown1	A7, A8, A9	1	364.36, 308.23, 402.91	-0.5	0.9
Ut -/- Neutr.A	A10, A11, A12	1	3553.95, 3909.05, 3669.48	59.5	3.2
Ut -/- Neutr.A	B1, B2, B3	1	4638.94, 3799.97, 4351.08	69.4	7.6
Ut -/- Neutr.A	B4, B5, B6	1	5194.78, 5084.52, 4555.35	81.5	6.1
Ut +/+ Neutr.	B7, B8, B9	1	3781.92, 3605.08, 3719.88	59.3	1.6
Ut +/+ Neutr.	B10, B11, B12	1	3555.91, 3854.61, 4312.17	63	6.8
Ut +/+ Neutr.	C1, C2, C3	1	4295.98, 3997.73, 4443.87	69	4.1
5nM -/- Neutr	C4, C5, C6	1	2762.12, 3128.66, 2936.44	45.7	3.3
5nM -/- Neutr	C7, C8, C9	1	3301.17, 3005.54, 3108.2	49.2	2.7
5nM -/- Neutr	C10, C11, C12	1	3192.86, 3109.57, ~~7406.46~~	49.5	1.1
5nM +/+Neut	D1, D2, D3	1	2989.98, 3066.42, 3278.31	48.8	2.7
5nM +/+Neut	D4, D5, D6	1	3351.52, 3730.43, 3680.82	57.3	3.7
5nM +/+Neut	D7, D8, D9	1	4084.13, 4011.98, 4343.28	67.3	3.1
10nM -/- Ney	D10, D11, D12	1	2426, 2903.81, 3192.44	43.9	6.9
10nM -/- Neu	E1, E2, E3	1	4205.49, 3850.84, 4337.19	67	4.5
10nM -/- Neu	E4, E5, E6	1	5600.21, 4427.18, 4585.76	80.2	11.4
10nM +/+Neu	E7, E8, E9	1	3065.91, 3020.79, 3392.55	49.6	3.6
10nM +/+Neu	E10, E11, E12	1	3430.01, 4196.13, 3912.81	61.9	6.9
10nM +/+Neu	F1, F2, F3	1	3669.13, 4116.1, 4613.08	67	8.4
20nM -/-Neut	F4, F5, F6	1	2346.72, 1960.03, 2229.73	32.1	3.5
20nM -/-Neut	F7, F8, F9	1	2087.88, 2341.23, 2147.68	32.3	2.4
20nM -/-Neut	F10, F11, F12	1	2648.28, 2484.46, 3770.97	46.2	12.5
20nM +/+Neu	G1, G2, G3	1	3640.59, 3377.18, 3082.26	53.3	5
20nM +/+Neu	G4, G5, G6	1	2771.68, 3242.11, 2694.14	45	5.3
20nM +/+ Ne	G7, G8, G9	1	2734.99, 2868.98, 2651.43	42.3	2

Raw data (in cpm) presenting cell lysis by CDC (in %) of bortezomib-treated Raji cells at 48h

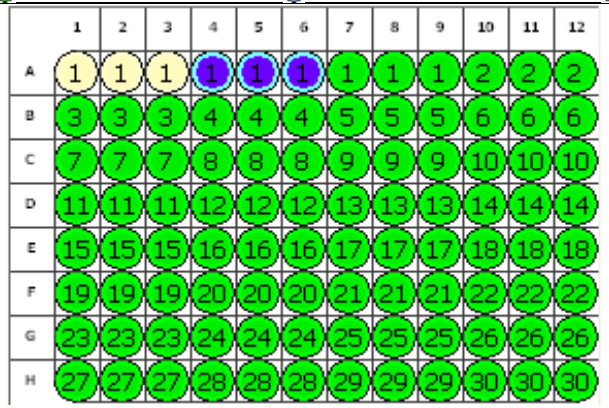
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 12: The raw data (in cpm) and the cell lysis by CDC (in %) of bortezomib-treated Raji cells at 48h. The impact of pre-treatment of tumor cells with bortezomib for 48h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	733.93, 686.17, 721.11	0	0.2
Pos Control1	A4, A5, A6	1	11617.5, 11736.2, 11130	100	3
Unknown1	A7, A8, A9	1	710.32, 628.8, 748.46	-0.2	0.6
Ut -/- Neutr.A	A10, A11, A12	1	6241.99, 5825, 6087.96	49.5	2
Ut -/- Neutr.A	B1, B2, B3	1	6917.34, 5816.53, 5859.82	50.9	5.8
Ut -/- Neutr.A	B4, B5, B6	1	6333.48, 7290.47, 7224.98	57.8	5
Ut +/+ Neutr.	B7, B8, B9	1	6582.97, 7356.94, 6211.71	55.7	5.4
Ut +/+ Neutr.	B10, B11, B12	1	5931.68, 6571.99, 6855.22	53.2	4.4
Ut +/+ Neutr.	C1, C2, C3	1	6546.13, 6327.89, 7199.03	55.4	4.2
5nM -/- Neutr	C4, C5, C6	1	6089.44, 6211.14, 6069.44	50.2	0.7
5nM -/- Neutr	C7, C8, C9	1	5951.88, 5420.62, 5858.37	46.7	2.6
5nM -/- Neutr	C10, C11, C12	1	5943.95, 5869.37, 8254.65	55.4	12.6
5nM +/+Neut	D1, D2, D3	1	5868.83, 7219.47, 7118.5	55.9	7
5nM +/+Neut	D4, D5, D6	1	7274.62, 6421.59, 6685.37	56.4	4.1
5nM +/+Neut	D7, D8, D9	1	7208.13, 7794.18, 8694.99	66.6	6.9
10nM -/- Ney	D10, D11, D12	1	7505.25, 8419.88, 7689.57	66.4	4.5
10nM -/- Neu	E1, E2, E3	1	7411.7, 6706.96, 6478.59	57.1	4.5
10nM -/- Neu	E4, E5, E6	1	7242.22, 8587.88, ~~21083.2~~	66.8	8.8
10nM +/+Neu	E7, E8, E9	1	8249.21, 8313.81, 8119.23	69.7	0.9
10nM +/+Neu	E10, E11, E12	1	6931.62, 8377.16, 8086.51	65.7	7.1
10nM +/+Neu	F1, F2, F3	1	9350.85, 8629.74, 10234.6	80.6	7.5
20nM -/-Neut	F4, F5, F6	1	6192.15, 6949.11, 6144.81	53	4.2
20nM -/-Neut	F7, F8, F9	1	6507.51, 6593.44, 6148.08	52.9	2.2
20nM -/-Neut	F10, F11, F12	1	6326.35, 6317.5, ~~16810.2~~	52	0.1
20nM +/+Neu	G1, G2, G3	1	7749.68, 7826.42, 7153.71	63.7	3.4
20nM +/+Neu	G4, G5, G6	1	6793.06, 6620.78, 6099.73	53.7	3.3
20nM +/+ Ne	G7, G8, G9	1	6882.27, 8131.92, 8929.04	67.4	9.6

Raw data (in cpm) presenting cell lysis by CDC (in %) of fludarabine-treated Raji cells at 24h

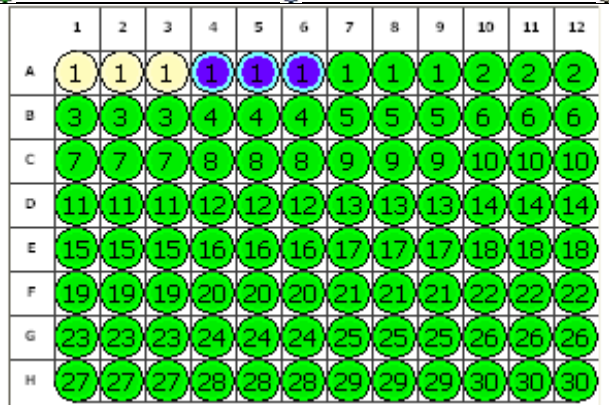
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

Table 12: The raw data (in cpm) and the cell lysis by CDC (in %) of fludarabine-treated Raji cells at 24h. The impact of pre-treatment of tumor cells with fludarabine for 24h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	707.52, 701.15, 687.01	0	0.2
Pos Control1	A4, A5, A6	1	5316.1, 5673.04, 5428.25	100	3.8
Unknown1	A7, A8, A9	1	587.46, 674.14, 681.74	-1.1	1.1
Ut	A10, A11, A12	1	3135.49, 3305.88, 3546.71	55.1	4.3
Ut	B1, B2, B3	1	3674.42, 3263.53, 3523.82	58.4	4.4
Ut	B4, B5, B6	1	3315.61, 3524.29, 3819.9	59.8	5.3
Ut + neut. Ab	B7, B8, B9	1	2380.16, 2853.3, 2590.95	40	5
Ut + neut. Ab	B10, B11, B12	1	2495.87, 2560.86, 2927.51	41.1	4.9
Ut + neut. Ab	C1, C2, C3	1	5675.51, 7596.74, 7552.11	130.8	23
1µM	C4, C5, C6	1	1458, 1636.94, 1751.1	19.2	3.1
1µM	C7, C8, C9	1	1775.26, 1903.21, 2096.17	25.7	3.4
1µM	C10, C11, C12	1	1955.14, 2192.48, 2441.24	31.4	5.1
1 µM + neut. A	D1, D2, D3	1	2203.94, 1939.09, 2241.25	29.9	3.5
1 µM + neut. A	D4, D5, D6	1	2368.25, 2290.63, 2313.46	34.1	0.8
1 µM + neut. A	D7, D8, D9	1	2379.47, 2385.14, 2495.6	36.1	1.4
3 µM	D10, D11, D12	1	1058.24, 1411.46, 1199.1	11	3.7
3 µM	E1, E2, E3	1	1484.21, 1290.41, 1519.71	15.4	2.6
3 µM	E4, E5, E6	1	1498.5, 1553.81, 1501.02	17.2	0.7
3 µM + neut.	E7, E8, E9	1	1651.47, 1991.48, 1650.73	22.3	4.1
3 µM + neut.	E10, E11, E12	1	1932.24, 1682.63, 1690.08	22.4	3
3 µM + neut.	F1, F2, F3	1	1785.23, 1580.46, 1531.1	19.6	2.8
5 µM	F4, F5, F6	1	923.72, 959.93, 957.69	5.2	0.4
5 µM	F7, F8, F9	1	895.9, 873.71, 1044.72	5	1.9
5 µM	F10, F11, F12	1	951.83, 1068.48, ~~2770.24~~	6.5	1.7
5 µM+ neut. A	G1, G2, G3	1	1140.42, 1033.02, 1004.83	7.6	1.5
5 µM+ neut. A	G4, G5, G6	1	1136.5, 1077.68, 1038.77	8.1	1
5 µM+ neut. A	G7, G8, G9	1	1282.15, 1255.27, 1485.75	13.5	2.6

Raw data (in cpm) presenting cell lysis by CDC (in %) of fludarabine-treated Raji cells at 48h

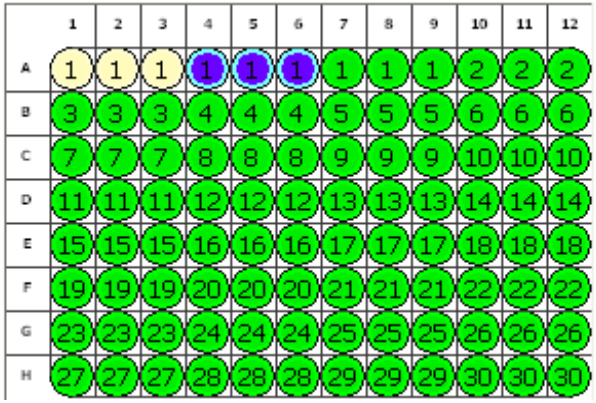
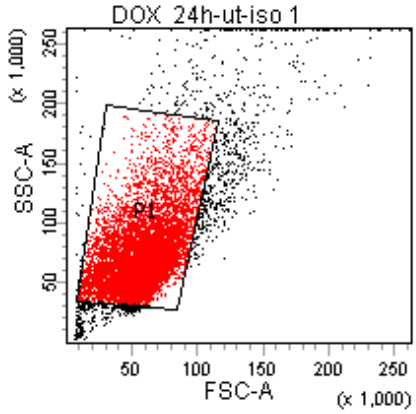
Assay Protocol Name:	51 Chrom Kifi
Readings:	Endpoint: Counts [12x8]
Well Types:	Unknown Pos Control Neg Control
Plate:	
Transformations:	Auto Flag By Well ("(pcv ([g]) > 25) and (x= furthest ([g]))") Normalise (Normalises Counts between Neg Control1.1 and Pos Control1.1) Standard Deviation (Calculates Standard Deviation of % specific lysis)

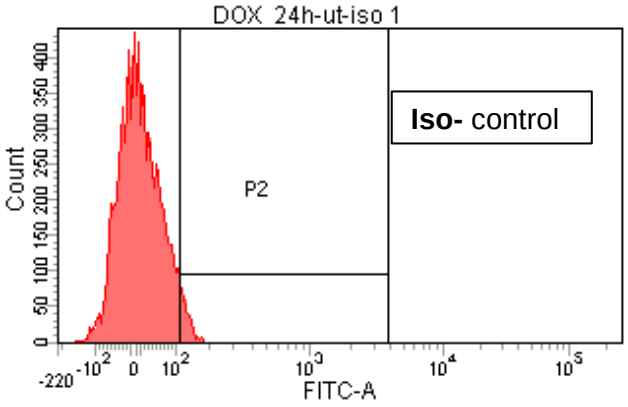
Table 13: The raw data (in cpm) and the cell lysis by CDC (in %) of fludarabine-treated Raji cells at 48h. The impact of pre-treatment of tumor cells with fludarabine for 24h on CDC either in absence or in presence of mCRP neutralising Abs was analyzed. Accordingly, the reference value (%) of the CDC was that of untreated cells. The percentage of specific lysis was calculated according to the following formula ((test release – spontaneousrelease) / (maximum release – spontaneous release)) × 100**.**

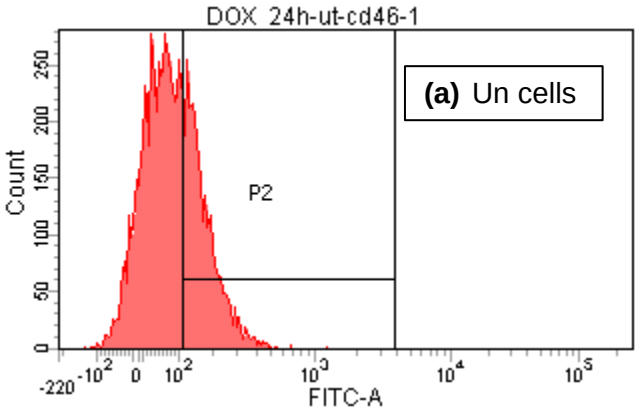
Group	Wells	Plate	Counts	% specific lysis	SD
Neg Control1	A1, A2, A3	1	641.64, 687.71, 699.58	0	0.5
Pos Control1	A4, A5, A6	1	5911.53, 6835.87, 6439.51	100	8.1
Unknown1	A7, A8, A9	1	649.07, 599.52, 685.87	-0.6	0.8
Ut	A10, A11, A12	1	3107.08, 3307.04, 3489.64	45.9	3.3
Ut	B1, B2, B3	1	3842.71, 3206.41, 3336.33	48.7	5.9
Ut	B4, B5, B6	1	3040.1, 3109.77, 3036.64	41.7	0.7
Ut + neut. Ab	B7, B8, B9	1	2467.06, 3001.24, 2903.42	37	5
Ut + neut. Ab	B10, B11, B12	1	2511.9, 2775.35, 2894.25	35.9	3.4
Ut + neut. Ab	C1, C2, C3	1	2840.17, 3141.82, 3384.86	42.8	4.8
1µM	C4, C5, C6	1	953.62, 1091.52, 1061.4	6.3	1.3
1µM	C7, C8, C9	1	979.99, 1017.86, 997.9	5.6	0.3
1µM	C10, C11, C12	1	1044.3, 1167.47, 1223.42	8.2	1.6
1 µM + neut. A	D1, D2, D3	1	1130.43, 1147.43, 1473.21	10	3.4
1 µM + neut. A	D4, D5, D6	1	1075.79, 1110.44, 1174.63	7.8	0.9
1 µM + neut. A	D7, D8, D9	1	1124.82, 1132.98, 1223.67	8.5	1
3 µM	D10, D11, D12	1	767.25, 883.28, 838.98	2.7	1
3 µM	E1, E2, E3	1	860.55, 911.19, 894.29	3.7	0.5
3 µM	E4, E5, E6	1	903.43, 910.22, 914.02	4.1	0.1
3 µM + neut.	E7, E8, E9	1	853.95, 921.51, 886.49	3.7	0.6
3 µM + neut.	E10, E11, E12	1	854.63, 832.11, 898.54	3.2	0.6
3 µM + neut.	F1, F2, F3	1	1003.88, 895.11, 892.22	4.4	1.1
5 µM	F4, F5, F6	1	587.57, 709.89, 640.56	-0.5	1.1
5 µM	F7, F8, F9	1	561.85, 574.6, 590.91	-1.8	0.3
5 µM	F10, F11, F12	1	610.66, 604.63, 893.48	0.5	2.9
5 µM+ neut. A	G1, G2, G3	1	651.21, 767.77, 636.7	0.2	1.3
5 µM+ neut. A	G4, G5, G6	1	614.96, 744.97, 735.81	0.4	1.3
5 µM+ neut. A	G7, G8, G9	1	739.19, 637.28, 693.29	0.2	0.9

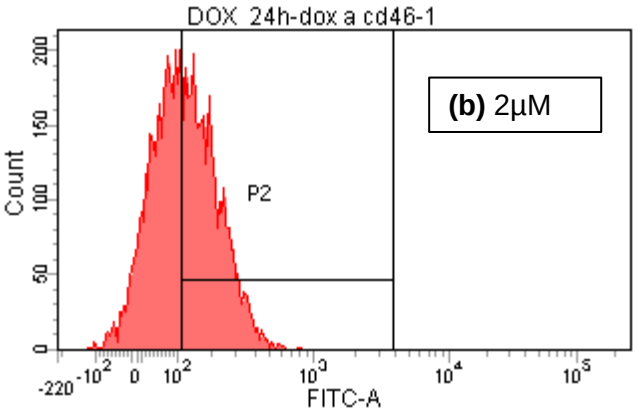
Representative histograms for expression of the mCRPs in treated SKBR3 cells with doxorubicin at 24h

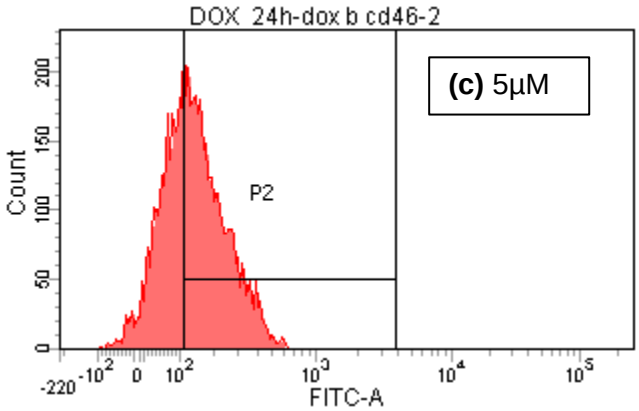
1. **Expression of CD46**

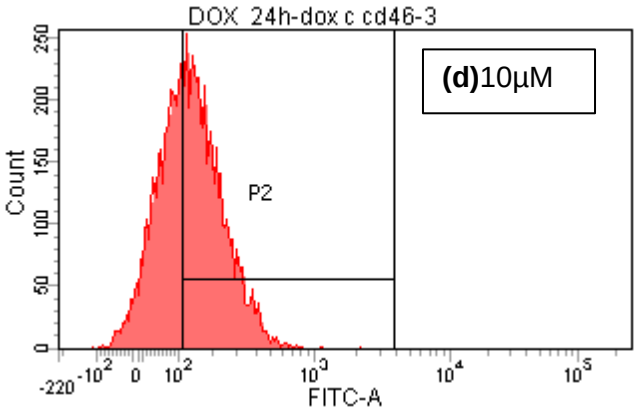




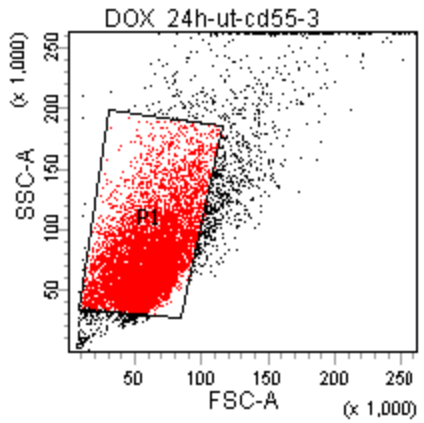


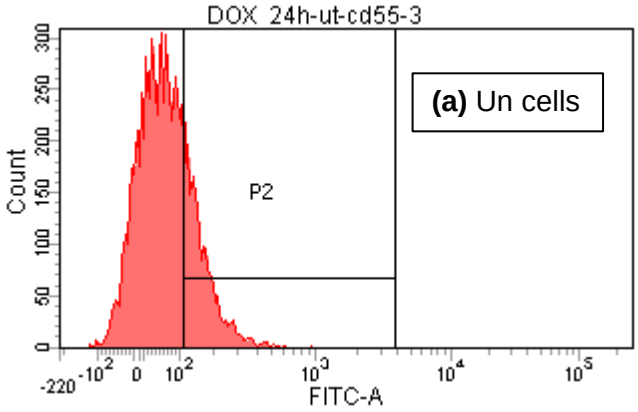


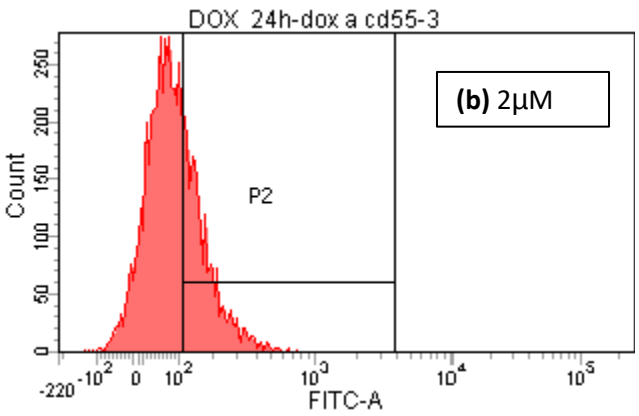


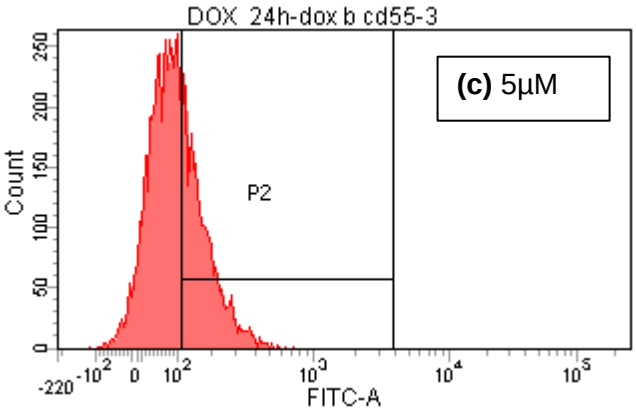


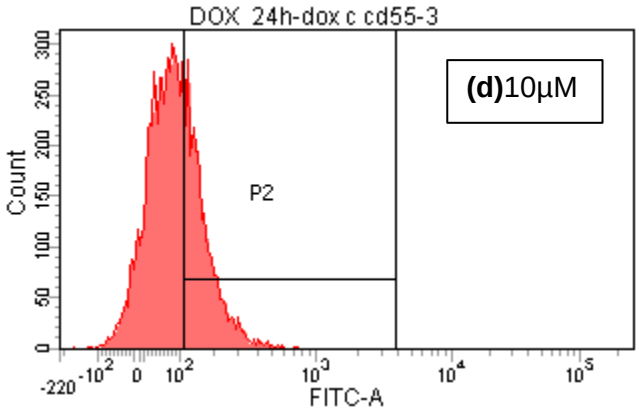
2. Expression of CD55



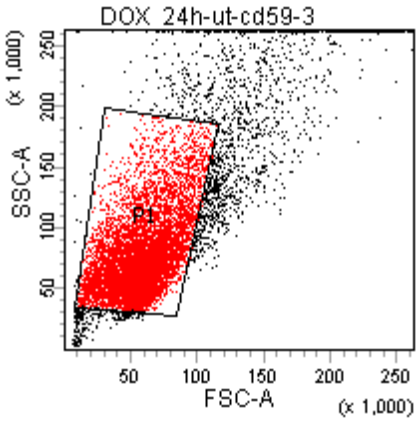


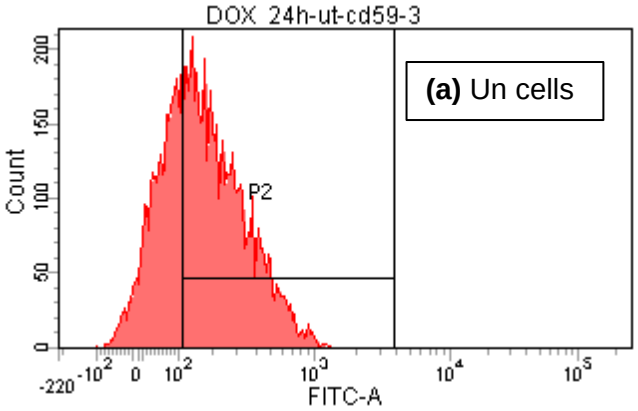


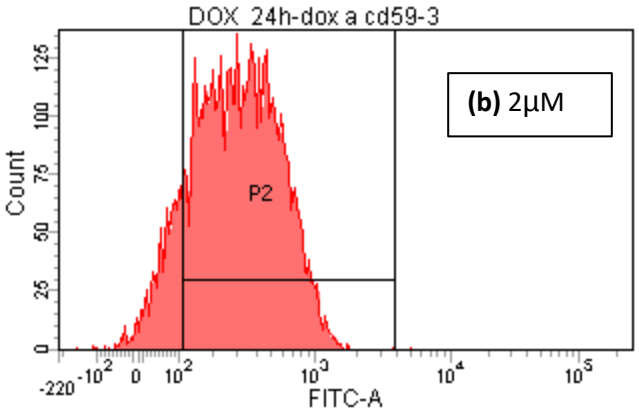


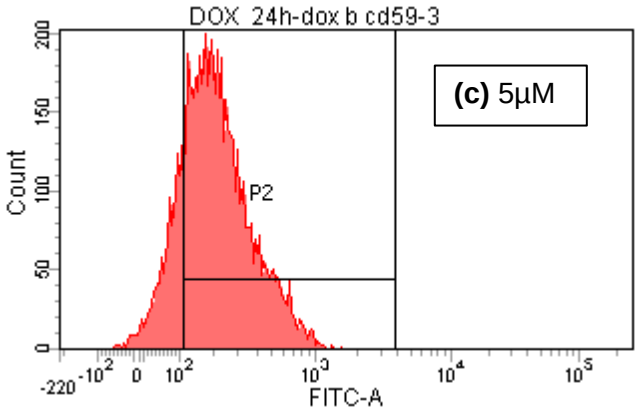


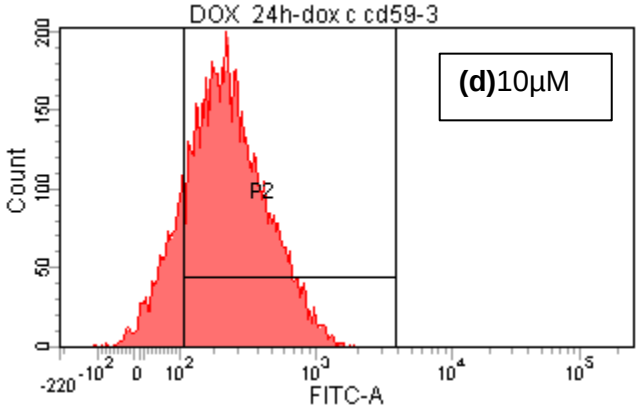
3. Expression of CD59





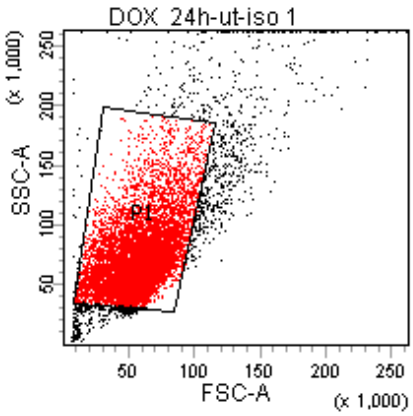


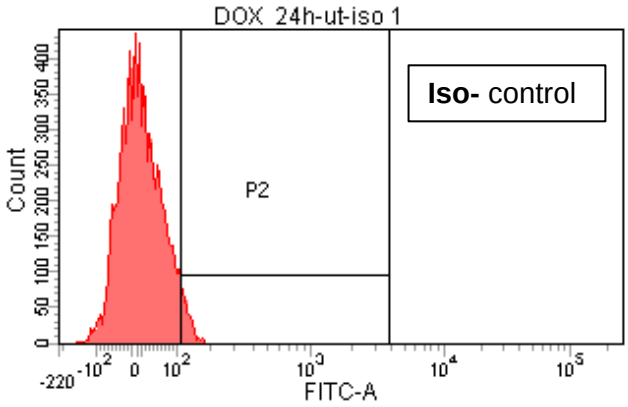


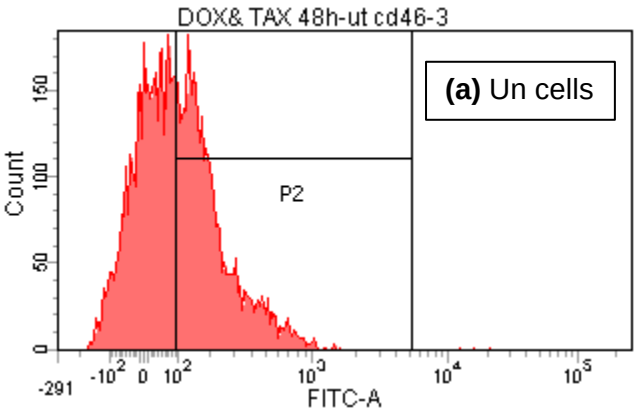


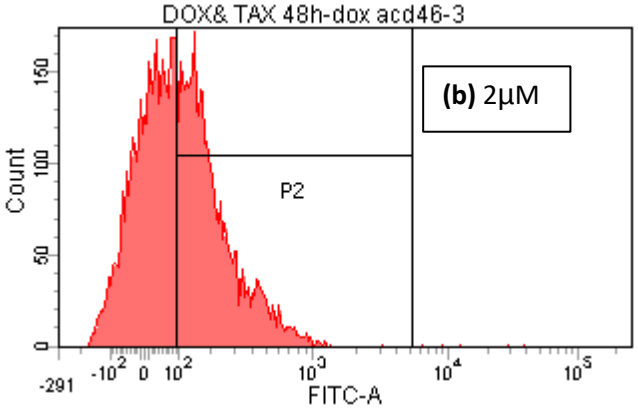
Representative histograms for expression of the mCRPs in treated SKBR3 cells with doxorubicin at 48h

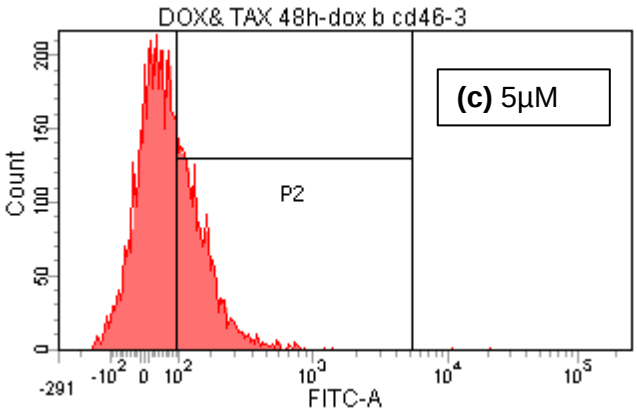
1. **Expression of CD46**

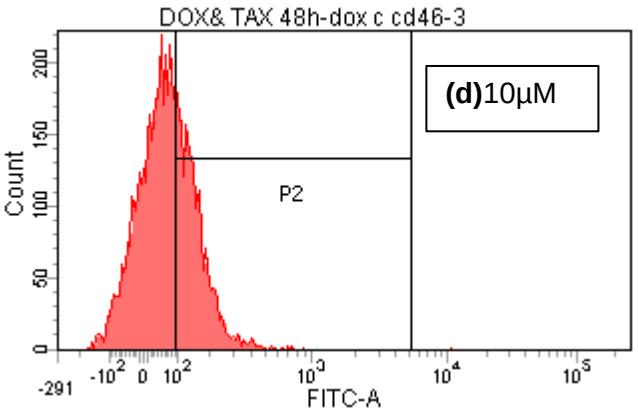




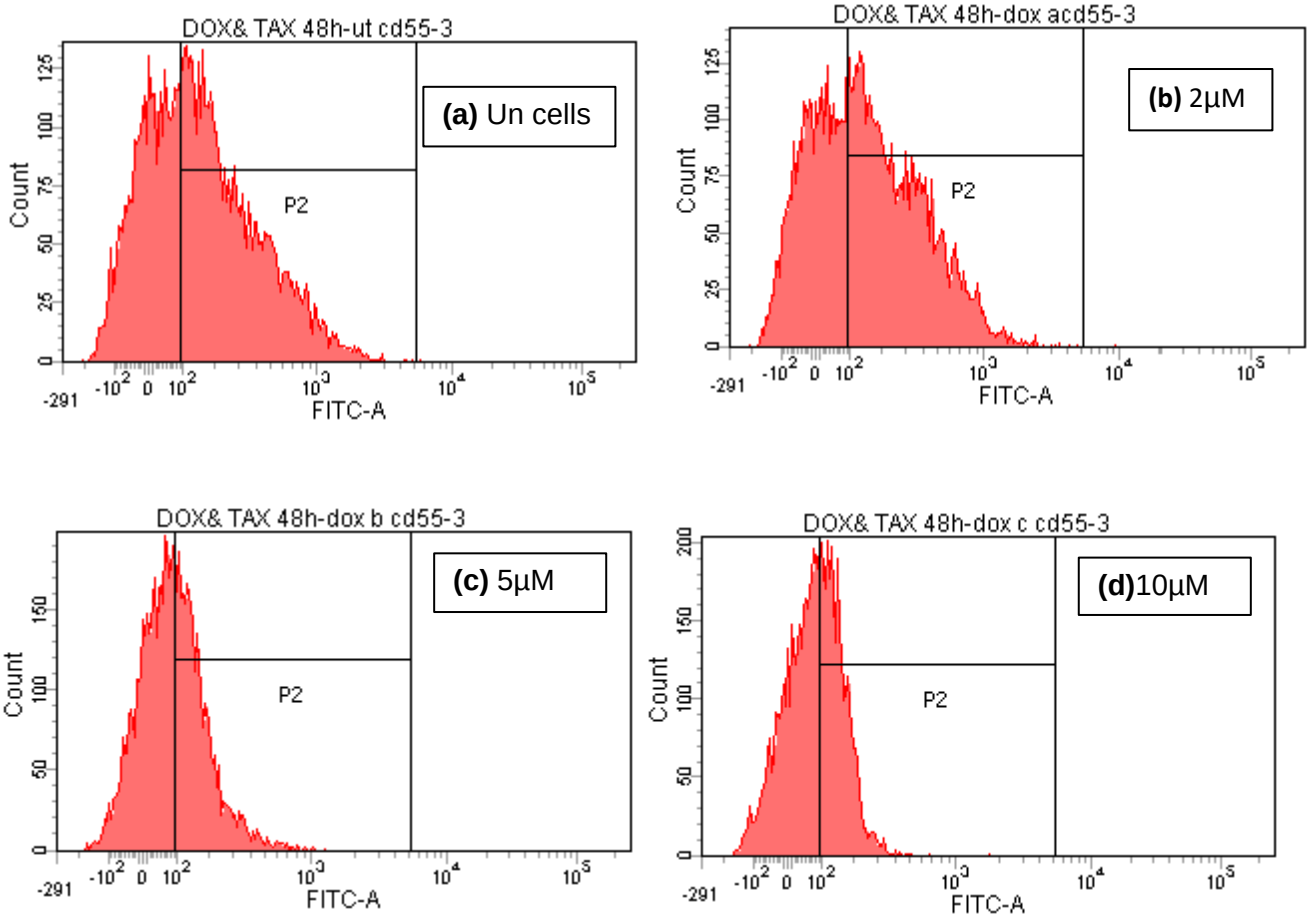




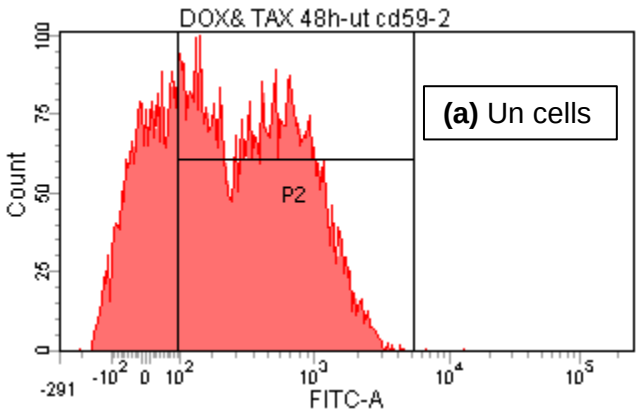


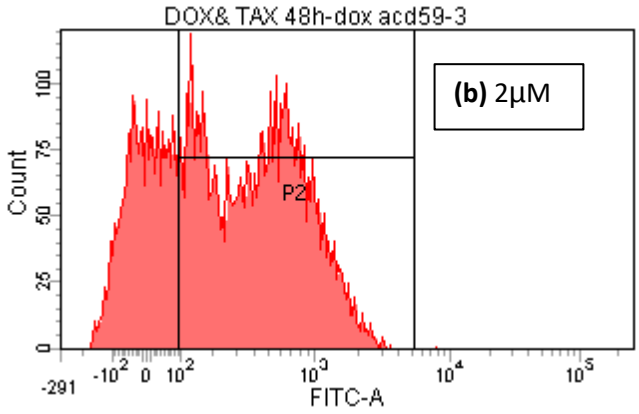


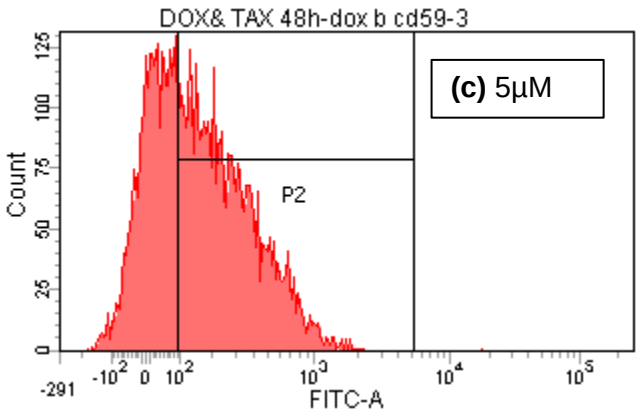
2. Expression of CD55

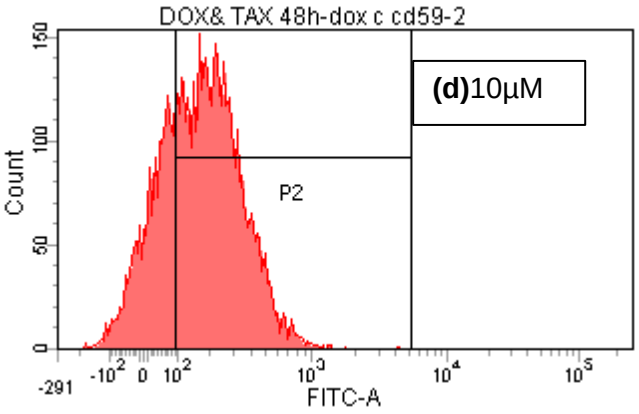


3. Expression of CD59



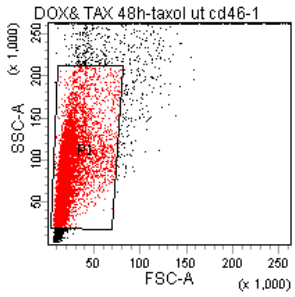


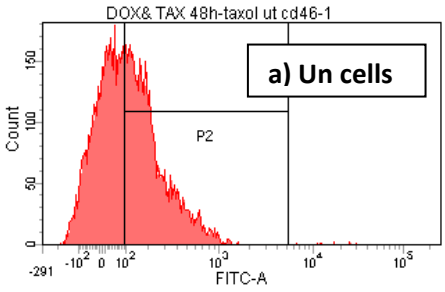


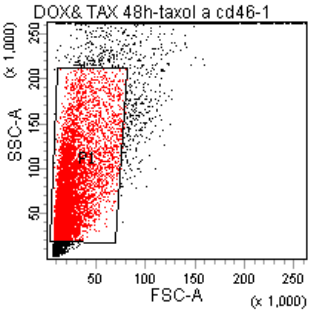


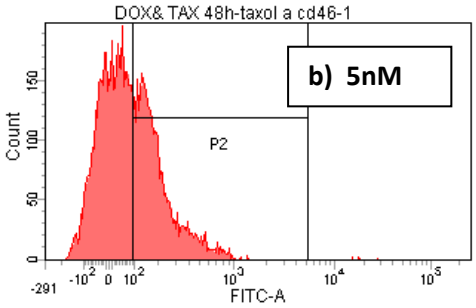
Representative histograms for expression of the mCRPs in treated SKBR3 cells with taxol at 48h

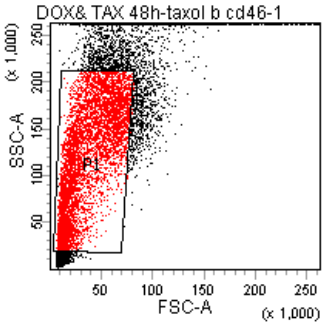
1. **Expression of CD46**

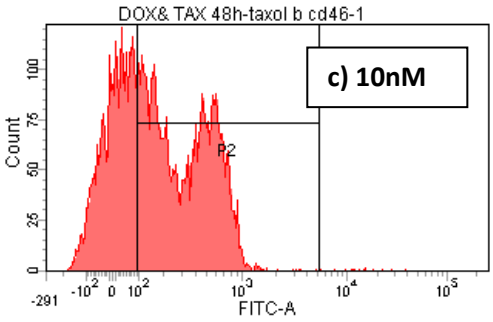


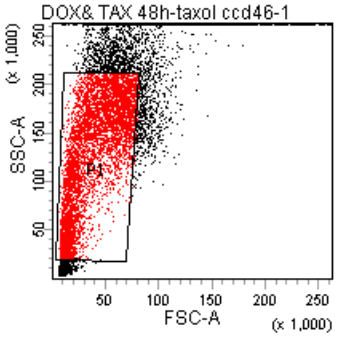


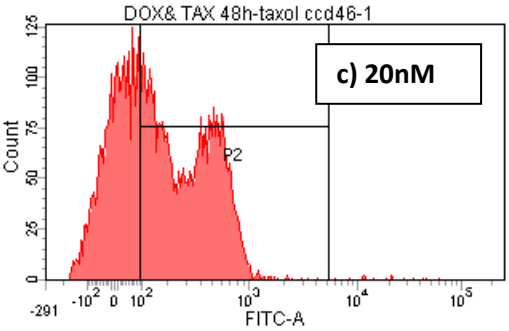




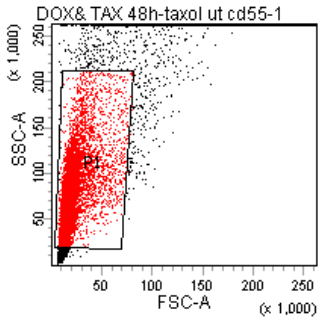


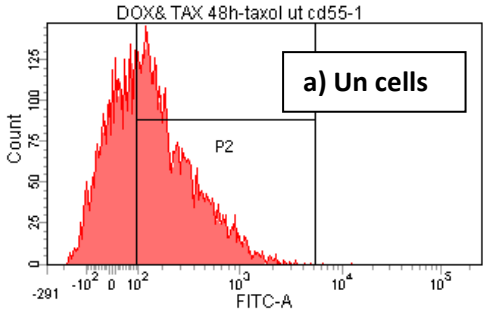


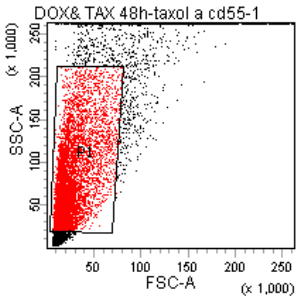


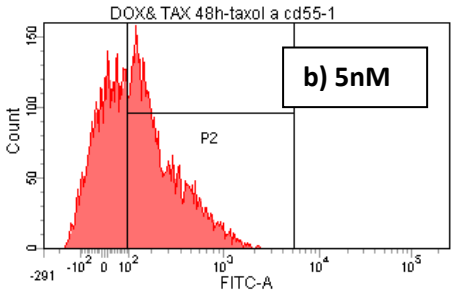


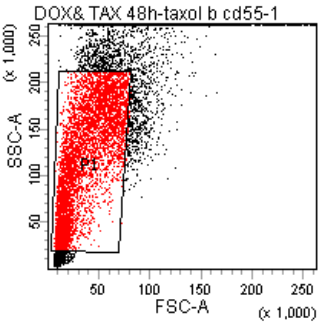
2. Expression of CD55

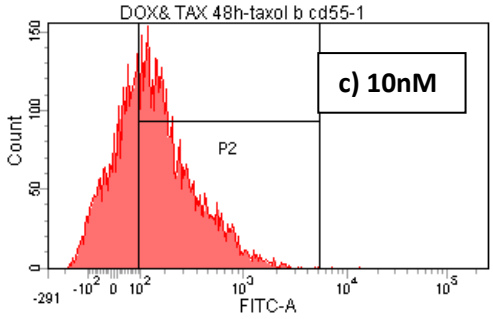


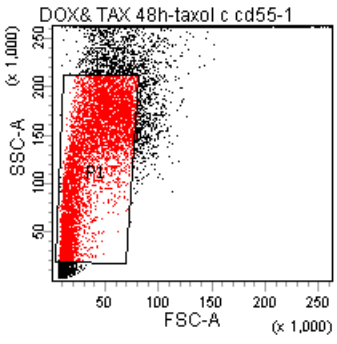


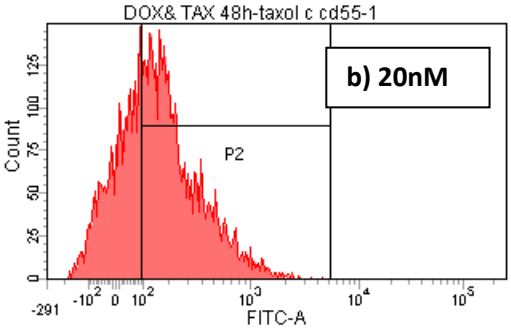




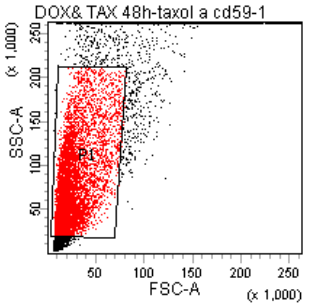


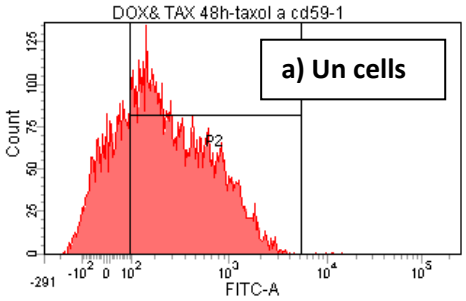


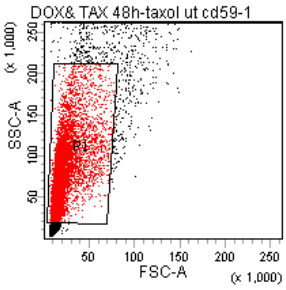


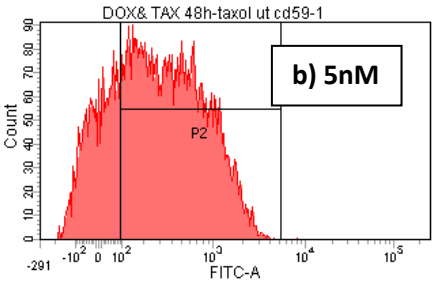


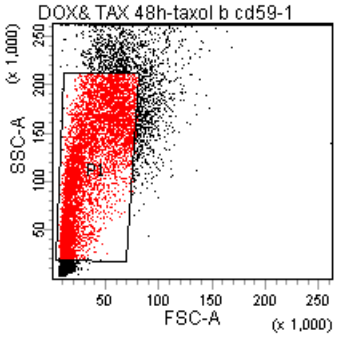
3. Expression of CD59

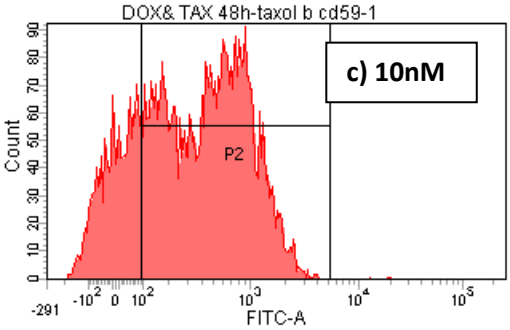


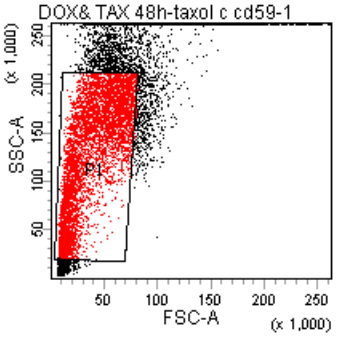


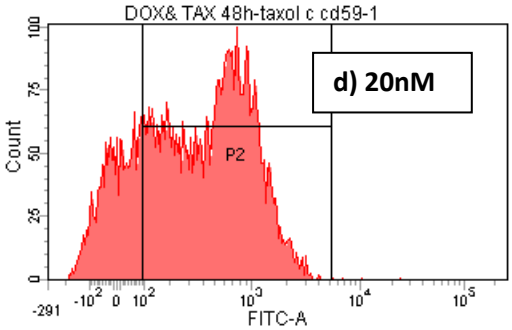




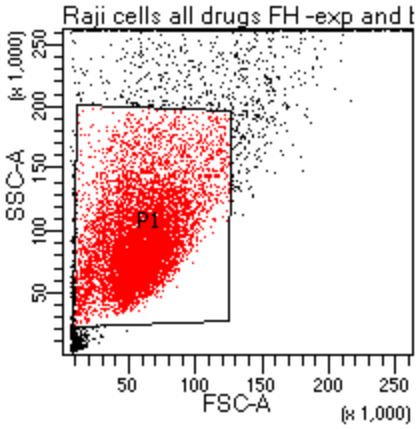


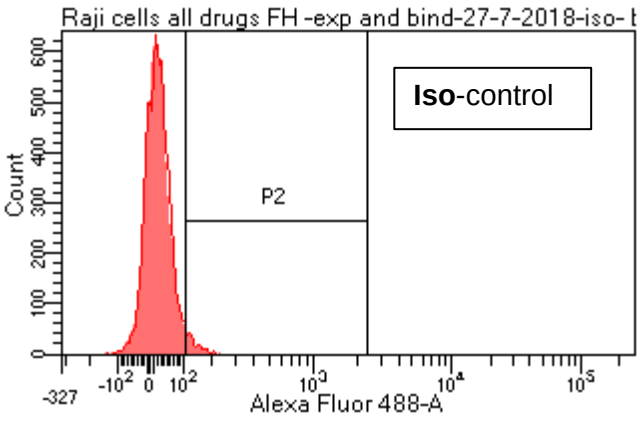


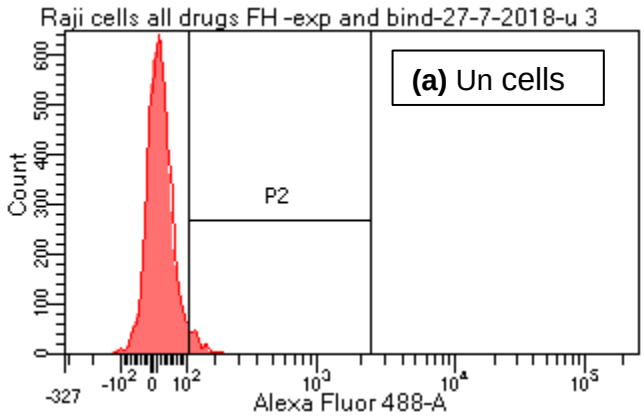


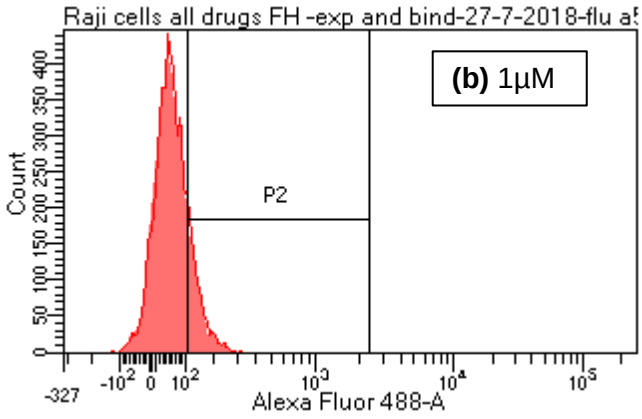


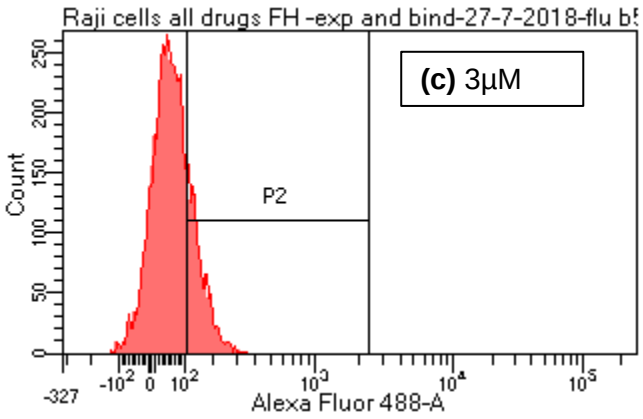
Representative histograms for fH binding to fludarabine-treated Raji cells at 48h

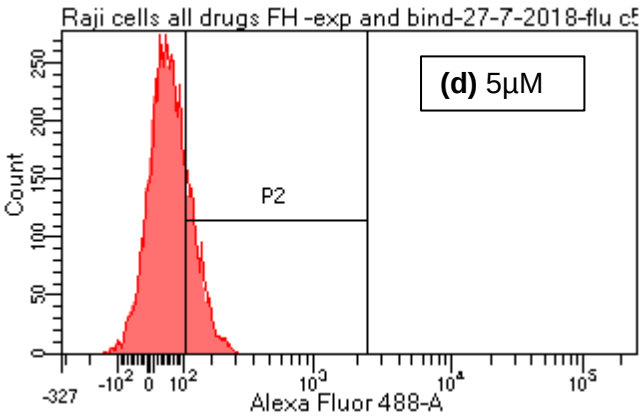




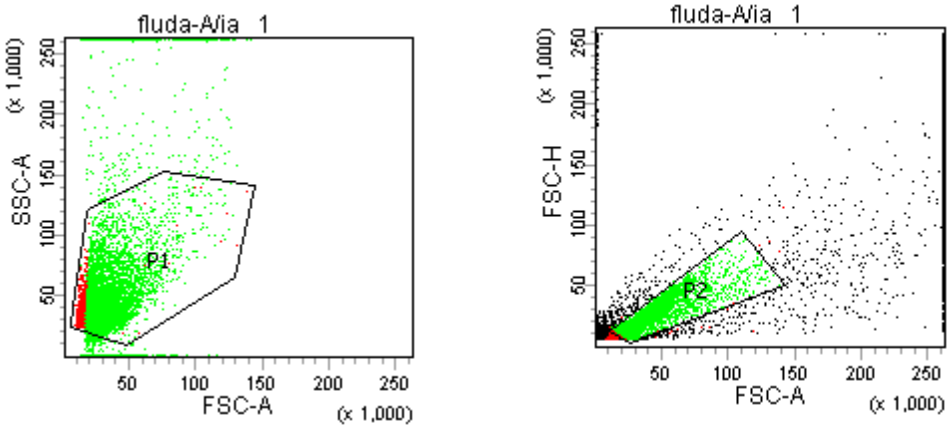


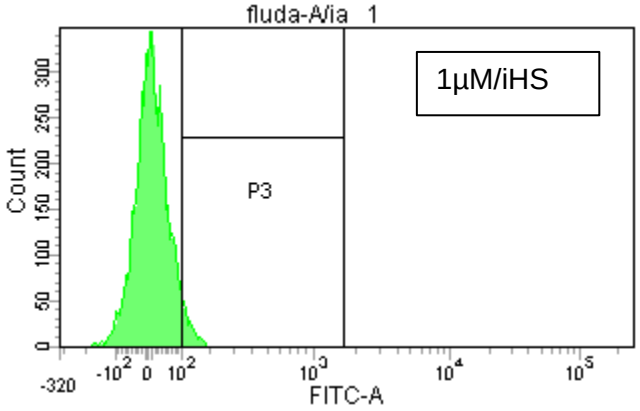


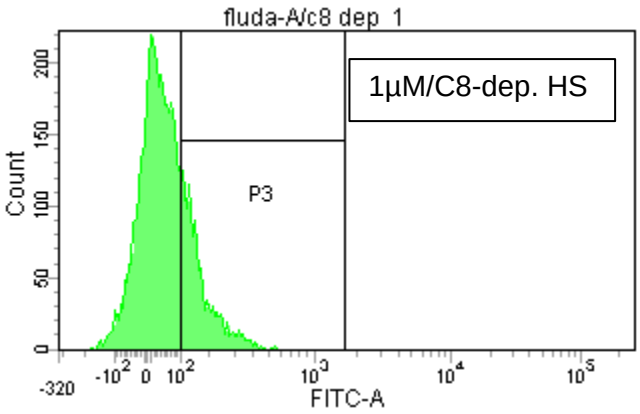


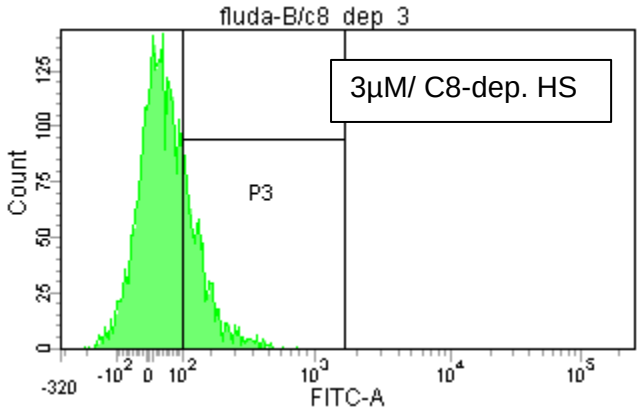


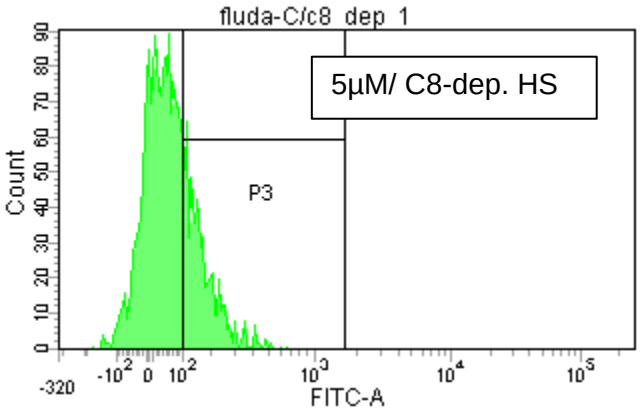
Representative histograms for opsonisation of fludarabine-treated Raji cells with iC3b at 48h






fluda-A/c8 dep 1
1µM/C8-dep. HS
P3
Count
FITC-A


fluda-B/c8 dep 3
3µM/ C8-dep. HS
P3
Count
FITC-A


fluda-C/c8 dep 1
5µM/ C8-dep. HS
P3
Count
FITC-A