UNIVERSITA' DEGLI STUDI DI PADOVA

DIPARTIMENTO DI SCIENZE DEL FARMACO

CORSO DI LAUREA MAGISTRALE IN CHIMICA E TECNOLOGIA FARMACEUTICHE

TESI DI LAUREA

CHARACTERIZATION AND CONJUGATION OF IgM WITH MONOMETHYL AURISTATIN E FOR PANCREATIC CANCER

RELATORE: Prof. Gianfranco Pasut
CORRELATORE: Dott.ssa Benedetta Campara, Prof. Polo Macor

LAUREANDA: Francesca Fatatis

ANNO ACCADEMICO 2023/2024

DEDICATION

Alla mia famiglia, agli amici e a me stessa

INDEX

Index

Index

ABBREVIATIONS

[ ], Concentration

A, Absorbance

AA, Amino acid

ACN, Acetonitrile

ADP, Antibody-Dependent Phagocytosis

AUC, Area Under the Curve

BCA, Bicinchoninic Acid Assay

BSA, Bovine Serum Albumin

CD, Cluster of Differentiation

CDC, Complement Dependent Cytotoxicity

Conc., Concntration

DAR, Drug Antibody Ratio

DMSO, Dimethyl sulfoxide

DTT, Dithiothreitol

EDTA, Ethylenediaminetetraacetic acid

EPR, Enhanced Permeability and Retention

Eq, Equivalents

ε, Molar extinction coefficient

Fc, Fragment Crystallizable

FPLC, Fast Protein Liquid Chromatography

Fragm, Fragmented

Gly, Glycine

Abbreviations

GPC, Glypican

GPI, Glycosyl-Phosphatidylinositol

GSH, Glutathione

Hc, Heavy Chain

HEPES, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid

HIC, Hydrophobic Interaction Chromatography

HPLC, High Performance Liquid Chromatography

IC, Inhibitory Concentration

IgG, Immunoglobulin G

IgM, Immunoglobulin M

IPA, Isopropyl alcohol

KCl, Potassium Chloride

KH_2PO_4, Potassium dihydrogen phosphate

Lc, Light Chain

Leu, Leucine

M, Molarity

mc-VC-PAB-MMAE, MaleimidoCaproyl-Valine Citrulline-para Aminobenzy

Loxycarbonyl-Monomethyl Auristatin E

MDR1, Multi Drug Resistance 1 (protein)

MeOH, Methanol

mg, milligrams

mL, milliliters

MMAE, Monomethyl Auristatin E

MMAF, Monomethyl Auristatin F

Abbreviations

mAU, milli-Absorbance Unit

MW, Molecular Weight

MWCO, Molecular Weight Cut Off

NaCl, Sodium Chloride

Na_2HPO_4, Sodium Phosphate Dibasic

pAcPhe, para-amino acetyl phenylalanine

PBS, Phosphate Buffer Solution

PES, Polyether sulfone

Pro, Proline

RP, Reverse Phase

Rpm, Revolutions per minute

Rt, Retention Time

RX, Reduction

SDS-PAGE, Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis

Sec, Selenocysteine

SEC, Size Exclusion Chromatography

SuO-VC-PAB-MMAE, N-hydroxy succinimidyl ester – Valine Citrulline- para

aminobenzyloxycarbonyl-monomethil Auristatin E

T, temperature

TCEP, Tris(2-carboxyethyl) phosphine

TFA, Trifluoroacetic acid

Thr, Threonine

TRIS, Tris(hydroxymethyl) aminomethane

TRS, Trastuzumab

Abbreviations

λ, Wavelength

ABSTRACT

Modern medicine allows to overcome multiple problems of traditional medicine for cancer treatment. These treatments consisted of using extremely toxic chemotherapeutic drug, that had various side effects. An example of a modern drug delivery system is ADC – Antibody Drug Conjugate. Generally, an ADC is composed by an antibody linked to a chemotherapeutic drug through a specific linker. The chemotherapeutic drug is extremely hydrophobic and toxic, meanly at systemic level, and for these reasons can't be administered by itself. The antibody and more specifically the linker increase the hydrophilicity of the drug and reduce the risk of systemic toxicity.

For my project, I used MMAE, Monomethyl Auristatin E, an inhibitor of tubulin polymerization, that prevent cell mitosis. This aspect is important because cancer is characterized by an increase in the tumour cell proliferation, that can lead to metastasis. In this way the result is a reduction in the proliferation and infiltration of the carcinogenic cells.

Usually, cancer is associated with overexpression of specific factors. The antibody is able to recognize these factors and to be directed only where these factors are overexpressed. When the antibody is linked to a chemotherapeutic drug, the conjugate will be directed only in the cancer microenvironment, where it will recognize selectively and with high affinity a receptor or an antigen usually expressed on the surface of the carcinogenic cells.

The aim of my thesis project is to formulate a new ADC, using an IgM as antibody. In particular, the IgM that I used was produced in murine cells, engineered against GPC-1 receptors supplied by the lab of Prof. Paolo Macor from the University of Trieste and research unit of Dott. Giuseppe Toffoli, IRCCS CRO Aviano.

I first characterize the IgM in order to determine the exact concentration and the main characteristics. Then I tried conjugating it with 2 different types of cleavable linkers, that can be cleaved only by specific enzymes (to obtain a DAR 20).

The linkers used were mc-Val-Cit-PAB-MMAE and SuO-Val-Cit-PAB-MMAE. The first one used to obtain cysteine conjugates and the other one to obtain lysine

conjugates. The cysteine conjugates were realized with a double step synthesis, composed by the reduction of the disulphide bonds step and the conjugation step. The lysine conjugates instead were realized with a single step synthesis, where only the conjugation step was required.

Two lysine conjugates and a cysteine conjugate at increasing concentration were tested in vitro on 2 different cell lines, GPC-1 positive BXPC3 cells (that grow for adhesion at the cell culture plate) and GPC-1 negative Jurkat cells (that grow in suspension in the cell culture). The cytotoxic effect (expressed through IC_{50} value) was evaluated at 48h using a CellTiter kit from Promega.

It was expected that the cysteine conjugate might have lost its activity on BXPC3 cell line because the reduction step, necessary for the drug coupling to the inter-chain cysteine could have reduced the entire antibody, inactivating it. Differently, for the lysine conjugates it was assumed a normal activity only on BXPC3 cell line, since the reduction step wasn't required for drug coupling.

Surprisingly, the results were totally different: the lysine conjugates didn't induced cytotoxicity on both cell lines instead the cysteine conjugate was active on both cell lines (higher on Jurkat cell line). These results showed at least one problem: the activity on Jurkat cell line, because it means that the activity of the ADC can be independent from GPC-1 expression.

For this thesis project I wanted to achieve an active targeting, since the IgM was specifically designed against GPC-1 receptors. What I obtained instead were ADCs that can eventually infiltrate in the tumour microenvironment through other mechanisms, such as EPR effect, due to the dimension of the conjugates and not for its active targeting recognition.

Another problem is the fact that, by reducing the IgM, its main function (CDC function) can be lost. This means that the activity of the cysteine conjugate is only related to the cytotoxic drug attached to it and it's not enhanced by the intrinsic function of the IgM.

1.0 INTRODUCTION

1.1 Cancer

Cancer is the second leading cause of death in the world. It can involve every tissue and organ of the body. It's characterized by an excessive and abnormal cell growth that can lead to the formation of entire mass of tumour all over the body (metastatic process)[1]. This type of cells is able to migrate distant from the original cancerous organ or tissue, through blood vessels and lymphatic fluids.

Cancer is also characterized by an alteration of the mechanisms that control the cell proliferation and the cell differentiation. All this together leads to an uncontrolled cells growth[2].

There is also an increase in the expression of proto-oncogenes and a decrease in the expression of anti-oncogenes. Proto-oncogenes are genes involved in the growth and proliferation of the cell and if they are overexpressed or mutated, they can lead to tumour formation (proliferation activity). On the opposite, anti-oncogenes are genes that blocks the activity of the proto-oncogenes and if their expression is lowered, the activity of the proto-oncogenes prevails (anti-proliferation activity).

The most common types of cancer are lung, stomach, prostate, liver, breast, cervical and colorectal cancer, with different incidence on men and women.

Cancer can be divided into 2 main categories:

- benign cancer -> noncancerous, not spreading type, generally removed surgically, it can become dangerous when it's located near critical structure (such as brain, trachea).

- malignant cancer -> cancerous, spreading type, different approach to remove it, with possible recurrence after surgery.

Cancer is not caused by a single factor but a combination of more factors. These factors can be genetic, environmental, or constitutional characteristics of the individual.

1.2 Cancer therapy

Nowadays, cancer is one of the major causes of death. Thanks to modern medicine, it was possible to alleviate the symptoms and extend the lifespan of oncological patients.

There are 5 strategies used to treat cancer[3]:

1. Surgery: surgery can be an option if the tumour is delimited to a precise area (ex: lung cancer, breast cancer) and if it has not metastasized. It's also the best choice for benign tumour.

2. Radiation: It's carried out with ionized radiation (mainly X rays) and it's a localized, non-invasive, pain free treatment. It induces the death of cancer cells by necrosis. Radiation is usually performed to reduce the dimension of solid tumours. Sometimes it's fully effective and it's able to eliminate the carcinogenic mass. It's usually associated with surgery and/or chemotherapy.

3. Chemotherapy: it uses drugs able to induce cancer cell death. It's the first line treatment for cancer. The chemotherapeutic drugs used depends on the type of tumour that must be treated. It's usually associated with radiation in order to reduce the dimension of the tumour mass and/or with surgery to eradicate the tumour mass. A chemotherapeutic drug acts inhibiting cancer cells proliferation by damaging the genetic makeup or by inhibiting the formation of the mitotic spindle. They usually lack selectivity: they act both on carcinogenic cells and on healthy cells. This represents one of the biggest disadvantages in the use of a chemotherapeutic drug, also because they are associated with a lot of side effects like nausea, fatigue, dizziness, myelosuppression, infertility, hair loss, infections, bleeding.

4. Hormonal therapy: it's used for types of tumours regulated by hormones, like prostatic and breast cancer. Generally, the growth of these tumours undergoes an increase in the release of specific hormones. Hormonal therapy allows to slow or stop the growth of the tumour mass by blocking the body's ability to produce these hormones or by interfering with the behaviour of

the hormones. Also hormonal therapy comes with side effects, because it can interfere with the hormonal balance.

5. Targeted therapy: it's the best treatment that a patient can receive. Targeted therapy is characterized by high specificity and high selectivity, and so the side effects are less than the other treatments. The aim is to target the specific tumour mass, without attacking healthy cells that don't need to be treated. This therapy is carried out by using a targeting agent that selectively recognize the antigen or allergen responsible of the tumour growth and induce the binding of the targeting agent to the specific receptor, preventing the binding of the antigen or allergen. All this to prevent the activation or deactivation of specific pathways that leads to cancer cells growth, proliferation, and metastasis.

Surgery and radiation can be classified as local treatments because they are localized, with the aim to remove the entire carcinogenic mass, instead chemotherapy, hormone therapy and targeted therapy as systemic treatments because the desired effect cannot be reached through local treatment.
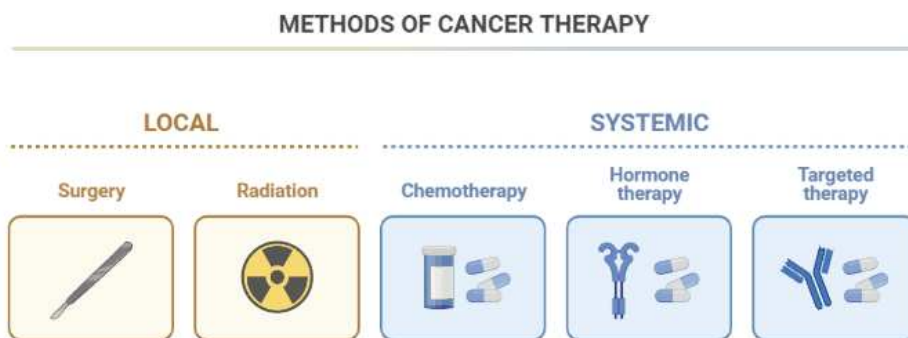

Figure 1.1: Cancer therapy: different approaches

1.3 Targeting types

There are 2 types of targeting: 1) Active targeting[4] and 2) Passive targeting[4].

1) Active targeting is characterized by the use of a directing or targeting agent. The aim is to direct the targeting towards only carcinogenic cell. Generally, a direct targeting agent can recognize a specific antigen or allergen express on the tumour cell surface responsible of the tumour growth and expansion. The aim is to prevent the activation or deactivation of specific pathways that leads to cancer cells growth, proliferation, and metastasis.

   Some advantages of this type of targeting are the reduction of the drug toxicity (If the drug reaches the bloodstream, it can cause systemic toxicity) and the increase of the specificity of the action. ADCs are the perfect example of active targeting: thanks to the Ab linked to the cytotoxic drug, it's possible to target only carcinogenic cells.

2) In this case there isn't a directing agent, so the action is not specifically on the cells, but it will occur on the tumour microenvironment. This is called 'EPR effect': Enhanced Permeability and Retention effect. Generally, a tumour expansion is characterized by an increase in the phenomenon of angiogenesis, new blood vessels formation. These vessels are irregular and fenestrated, and this leads to an increase of vessels permeability. The disadvantage of the EPR effect is that the drug can circulate longer and can infiltrate, facilitating the formation of metastases, and it can also attack healthy cells increasing the systemic toxicity.

1.4 ADC: Antibody Drug Conjugates

A direct application of the targeted therapy is the use of ADCs[5]. An ADC is a modern drug delivery system made of an antibody and a chemotherapeutic drug joined by a linker. It can be considered a pro-drug. The main aspect about ADCs is their selectivity and specificity. All this is carried out by the antibody, that is able to recognize a factor or antigen overexpressed only in the tumour microenvironment.

The advantage of the ADC is the fact that allows to bind a chemotherapeutic drug that generally cannot be used alone, due to its hydrophobicity and toxicity. The chemotherapeutic drug most used are inhibitors of tubulin polymerization, inhibitors of microtubules depolymerization and DNA damaging agents. The final effect will be the arrest of the cancer cells proliferation and in some cases also the regression of the tumour mass.

Generally, a cytotoxic drug is characterized by a very narrow therapeutic window because the difference between minimum effective dose and maximum tolerated dose is low. Binding these drugs to an antibody ensure the increase of the therapeutic window. This allows to increase drug delivery to the tumour mass (and so the dose needed is lower) and in the same moment reduce healthy-tissue exposure. The linker is the fundamental unit of the ADC because it allows to link the drug to the antibody and also because it increases the hydrophilicity of the drug and reduce its toxicity.
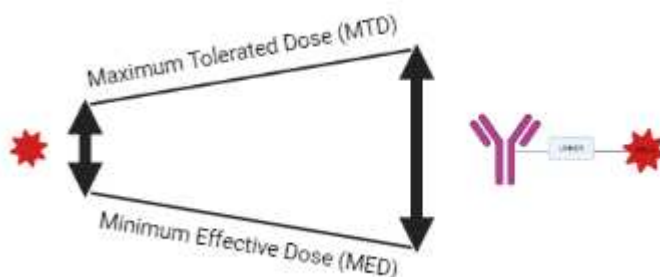
Figure 1.2: Graphic representation of the widening of the therapeutic window. Imagine created with Biorender.com.

The theory underneath the ADC concept comes from Paul Ehrlich. In 1913 he described the 'Magic bullet'[6] theory for cancer therapy. According to his theory, antibodies have the ability to recognize with high affinity only specific antigens and to act only where these antigens are overexpressed (tumour microenvironment). So, these antibodies can be considered as 'magic bullets' because they only act in the tumour, where the antigens level is higher. This theory is the basis of targeted therapy.

The first ADC was made in 1958 and it has methotrexate as chemotherapeutic drug linked to an antibody (IgG). Other cytotoxic drugs used were maytansines (as DM1, DM4) and auristatins (MMAE, MMAF) as tubulin polymerization inhibitors and calicheamicin and doxorubicin as DNA damaging agents.

Since 1958, a lot of ADCs were produced and approved by FDA (Food and Drug Administration). The first one was Mylotarg. The antibody used was Gentuzumab Ozogamicin (against CD33 expressed in leukaemia cells) and the drug was Calicheamicin. The number of ADCs approved by FDA is still very low but there are multiple new ones in clinical trials. This means that interest on this specific field of medical research is rising.
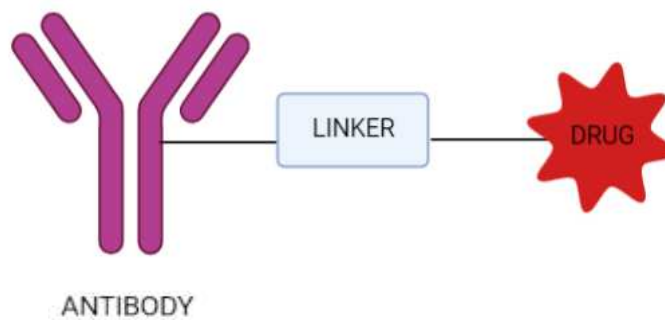
Figure 1.3: Representation of an ADC. Imagine created with Biorender.com

1.4.1: Cytotoxic drug

As I previously said, the drug used is generally extremely potent but also extremely toxic, with a very low therapeutic index. A low therapeutic index means that the risk associated with the use of this drug is higher than the benefit that comes from its use. There are other characteristics that are essential for a drug in order to be used in an ADC formulation[7]. I) The drug must be highly potent. This allows to attach less drug molecules to the antibody. The optimal number of drugs for antibody is usually 2-4. II) The drug shouldn't be immunogenic, so it shouldn't activate the immune system when injected. This is important because otherwise the action

and so the effect of the ADC are compromised. III) The drug should have a known mechanism of action. It's essential in case of immunological reaction. IV) The drug must have specific functional groups that allow the binding with the linker to the Ab. For this reason, it should be bifunctional to attach on one side the drug and on the other side the antibody. V) Potency and effect must be maintained after conjugation. It's important that the potency of the drug in an ADC is the same that as a free drug in order to evaluate the exact dose needed.

One of the biggest challenges in the formulation of an ADC is the hydrophobicity of the drug used. Usually, all these drugs have a very high hydrophobicity, that can be reduced thanks to the linker and Ab. This is a disadvantage because it's known that hydrophobic molecules are the best substrate for the efflux pumps expressed in cancer cells, as for example MDR1 protein. This protein is responsible for ADCs resistance phenomenon because if the drug is recognized by these pumps, the entire ADC will be recognized consequently, and it will be eliminated.

These drugs are characterised by two main mechanisms of action. I) Block of the cellular cycle in G2/M phases and apoptosis of the carcinogenic cell. Tubulin polymerization inhibitors are the main responsible for this action. Example: Auristatins derivates such as MMAF (Monomethyl Auristatin F) and MMAE (Monomethyl Auristatin E) and Maytansines derivates such as DM1 and DM4.

II) DNA damaging agents. They are generally molecules that damage DNA by crosslinking or alkylating. Examples: Calicheamicin and derivates and doxorubicin.
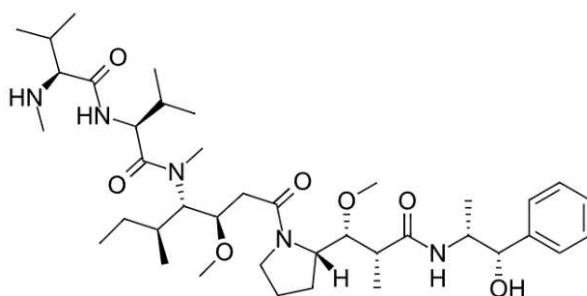

Figure 1.4: Representation of Monomethyl Auristatin E

1.4.2: Antibody

Antibodies, also known as Immunoglobulins, are glycoproteins that are produced and released by B lymphocytes when they interact with antigens. Antibodies are associated with a specific antigen[8]. After the binding of the antibody to the antigen, this complex can be eliminated. The aim in fact is to recognise a foreign molecule, such as an antigen, and to eliminate it.

Antibodies stimulate the humoral immune system, a part of the adaptive immune system, that is activated in the presence of a danger.

Every antibody has a characteristic Y shape, made of 2 Fab regions (Fragment Antigen Binding) and 1 Fc region (Fragment crystallisable)[9]. The Fab region is the region responsible of the biding of the antigen instead the Fc region is responsible for the activation of the immune system thanks to the interaction with Fc receptors and some proteins of the complement system. The Fab region has 2 variable domains and 2 constant domains. Every variable domain contains 3 CDR regions that guarantee the recognition of different epitopes that belong to different antigens. The constant domains instead have a stability and structure function. In general, the antibody consists of 2 light chains and 2 heavy chains. The light chains consist of polypeptides of the size of 22 kDa instead the heavy chains consist of polypeptides of the size of 50 kDa. Every heavy chain is made of 3 constant domain and 1 variable domain. Every light chain is made of 1 constant domain and 1 variable domain. A light chain and a heavy chain of a Fab region are connected by a disulphide bond instead the 2 heavy chains of the Fc region are connected by 2 disulphide bonds.
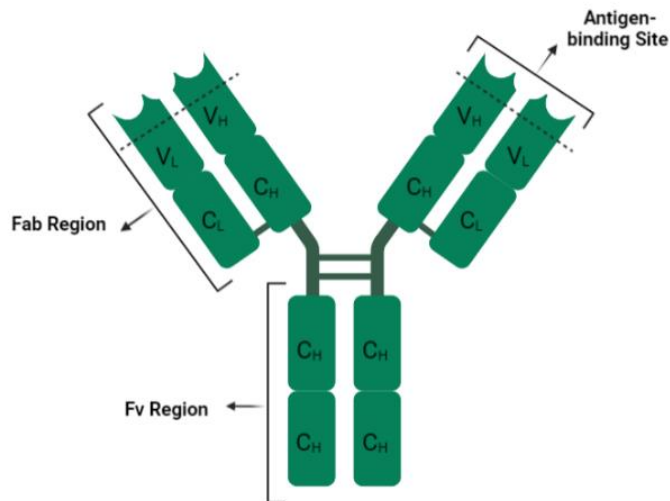
Figure 1.5: Antibody structure. Imagine created with Biorender.com

There are 5 different Immunoglobulins classes, that depends on the AA sequence of the constant domains: IgA, IgD, IgE, IgG, IgM. The characteristics are summarized in Table 1.

Antibody	Abundance	Type of Hc	Subclasses	MW
IgA	13 %	α	2	385 kDa
IgD	1%	δ	/	180 kDa
IgE	0,002%	ε	/	200 kDa
IgG	80 %	Γ	4	150 kDa
IgM	6 %	μ	/	900 kDa

Table 1: Summary of the Immunoglobulin classes, with the main characteristics.

Serum IgM can be pentameric or hexameric. The pentameric form it's made of 5 Immunoglobulin G (~180 kDa) held together by a J chain (~15 kDa). The hexameric form instead it's made of 6 Immunoglobulin G. It was demonstrated that the J chain is not present in this specific form. Each monomers have 2 Antigen-binding sites for a total of 10 or 12 possible antigen molecules attached to.

Figure 1.6: IgM Structure. Light chains (green) and Heavy Chains (blue).
Antigen binding sites (red). Imagine created with Biorender.com

1.4.2.1: Mechanism of action IgM-mediated

Natural IgM antibodies are responsible for the "Innate Immunity"[10]. They represent the majority of the secreted IgM antibodies, found in serum.

It's not totally clear where they are produced, but the principal option is the B-1 cells. They are produced in response to antigen exposure or neo-epitopes on dying c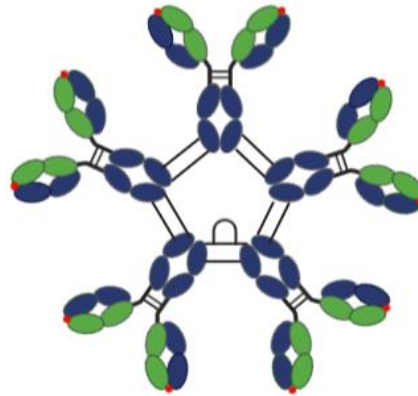ells. Some antigens specifically recognised by natural IgMs are phosphorylcholine, phospholipids, lipopolysaccharide, and others.

They are also important for immunological tolerance and immune regulation. They are responsible for the agglutination process: due to their dimension, this process is amplified. IgMs can recognize different epitopes and form a complex that can be easily eliminated by complement fixation or by macrophages.

These natural IgMs use low affinity binding to similar antigens, and their ability to eliminate these antigens is increased by having 10 (for the pentameric form) or 12 (for the hexameric form) binding sites.

IgMs can also have an effector function, through CDC activity. When IgMs are bounded to the antigen surface on the target cell, the complement system is activated thanks to the simultaneous binding of the IgMs to protein C1q. This protein is the first protein needed to initiate the complement system. The formation of this complex induces the lysis of the target cell, where the antigen is attached to.

Another mechanism associated with IgMs is ADP, Antibody-Dependent Phagocytosis. In this case, IgMs bind to the antigen and, through the FcγR, bind also to macrophages, inducing the phagocytosis process that leads to complex elimination.
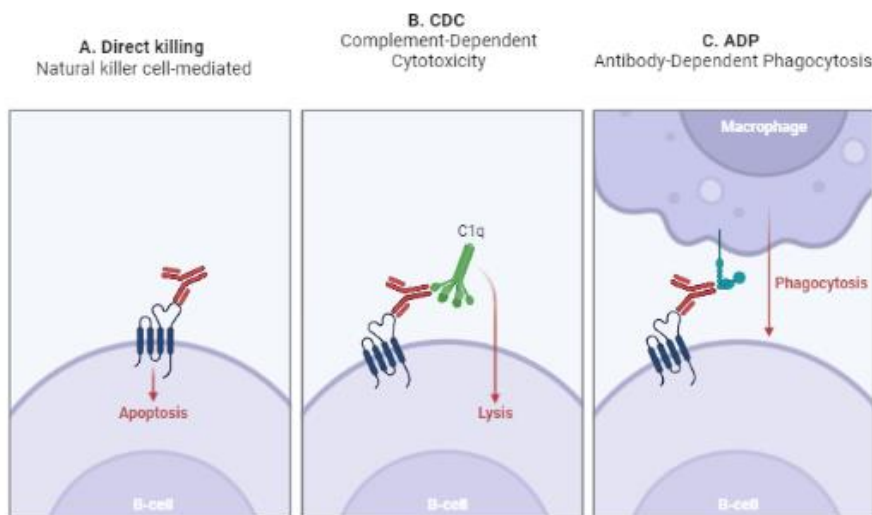
Figure 1.7: IgM mechanisms of action: Direct killing, Complement-Dependent Cytotoxicity and Antibody-Dependent Phagocytosis. Imagine adapted from Biorender.com

1.4.3: Linker

The linker is a fundamental unit of an ADC. It allows to link the drug to the antibody[11]. Another function of the linker is to increase the hydrophilicity associated with the drug. Generally, the linker should be bifunctional: on one side there is the binding of the cytotoxic drug and on the other one the binding of the antibody. In addition to that, these 2 reactive groups should be orthogonally reactive. The most common functional groups are cysteine and lysine residues on the Ab and activated carboxylic groups, isothiocyanate and maleimide for the drug.

The linker can also be not cleavable or cleavable. The not cleavable linker is a linker that generally doesn't release the drug in normal conditions. The release occurs thanks to the acidic pH in the lysosomes, by some enzymes that are active only at

acidic pH (pH in the lysosomes: ~ 5; pH in the blood: ~ 7.4). The release is not mediated by cleavage enzymes.

The cleavable linker instead is a linker that can be hydrolysed by specific enzymes. The most common enzyme is the cathepsins B, a cysteine protease overexpressed in the extracellular environment in many cancers.

Both linkers have advantages and disadvantages. The not cleavable linker has the advantage of high stability in the bloodstream. In fact, this linker is stable at physiological pH (bloodstream) and instead can be cleaved at acidic pH (lysosome of the tumour cell). A disadvantage can be the fact that the entire ADC must be internalized into the tumour cell in order to release the drug. This can be difficult due to the big dimension of the ADC. Another disadvantage can be the fact that the drug released has a residue of the linker still attached to it. To avoid this, a traceless linker or a self-immolative spacer can be used. It's also important that the drug released is still active in order to exploit its function. For this reason, the activity of the linker + drug should be studied previously in order to verify that it's the same.

The cleavable linker has the advantage of avoiding the internalization of the entire ADC in the tumour cell because the drug can be released also in the tumour microenvironment and so only the drug will be internalized. These linkers are cleaved in response to differences between extracellular and intracellular environments (for example differences in the level of pH, ROS or glutathione) by specific enzymes that are overexpressed in the tumour (for example cathepsin, plasmin and β-glucuronidase).

The biggest disadvantage is the fact that the drug can be released in the bloodstream, before arriving in the tumour microenvironment.

Generally, a cleavable linker is better than a not cleavable one, but all depends on the stability of the conjugate in the bloodstream.

There are different types of cleavable linker that allow to have a site selective drug release.

I) Labile Acid Linker. This kind of linker can be easily hydrolysed in acidic environment, such as endosomes and lysosomes. The main functional group is the Hydrazone.

Example: Gentuzumab Ozogamicin. Its commercial name is Mylotarg. This Ab

is directed against CD33 overexpressed on the surface of leukemic cells. The drug attached is Calicheamicin (DNA damaging agent). The linker is cleavable in 2 different point: hydrazone group (acidic pH) and disulphide bond (by GSH).

II) Linker with disulphide bonds. This linker is sensitive to thiol groups.

For example, Glutathione is able to reduce the linker and to release the drug. The release occurs in the cytosolic environment because the concentration of free thiols (RX GSH) is higher than in the bloodstream. Also, the level of GSH in the tumour environment is higher than in normal tissue.

Example: Gentuzumab Ozogamicin.

III) Enzyme-cleavable linkers. These linkers can be cleaved by specific enzymes.

One example can be Cathepsin B that specifically recognizes Val-Cit dipeptide (Valine-Citrulline). In this case the release is site-specific because cathepsins B are usually overexpressed in the tumour microenvironment. Sometimes Phe-Lys dipeptide can be used.

Example: Brentuximab Vedotin. Its commercial name is Adcetris. This Ab is directed against CD30 overexpressed in Hodgkin's lymphoma and large-cell lymphoma cells. The drug used is MMAE. The linker instead contains a dipeptide (Val-Cit) that is cleaved by Cathepsin B.

Another example can be linkers cleaved by glycosidases, such as β-glucuronidase and β-galactosidase[12], that can be secreted by tumour cells. For example, β-glucuronidase catalyses the breakage of β-glucuronic acid residues into polysaccharides at lysosomal pH (acidic pH).
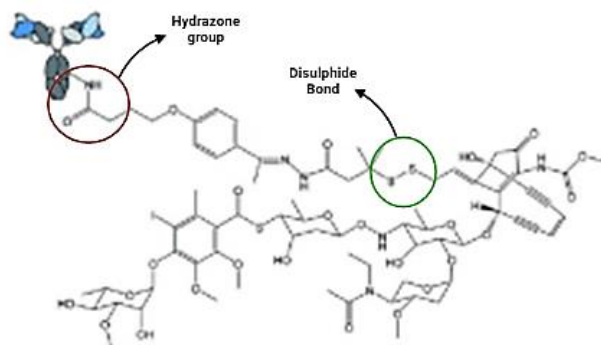
Figure 1.8: Gentuzumab Ozogamicin: cleavable linker with a hydrazone group (acid labile). Imagine modified with Biorender.com
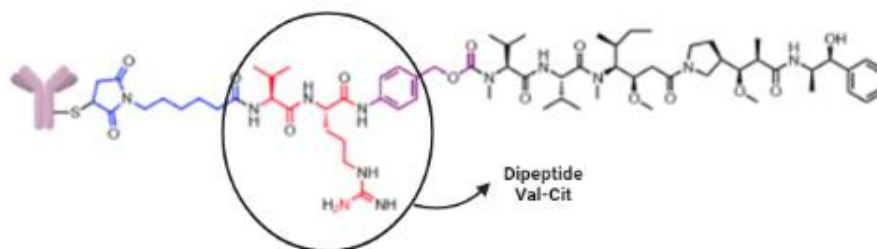
Figure 1.9: Brentuximab Vedotin: cleavable linker with a dipeptide (Val-Cit).
Imagine modified with Biorender.com
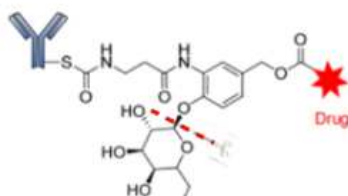
Figure 1.10: β-galactosidase cleavage site. Imagine modified with Biorender.com

1.4.4: Conjugation strategies

There are different approaches for the conjugation[13].

- Random coupling. This type of conjugation is possible through the amino group of the lysine of the Ab. It's random because it can occur on every amino group. Generally, the most exposed at the solvent (ε amine group) are the one that can be easily attached. In order to force the binding on the external lysine, the pH of the reaction should be set around 8, which is the pKa of the ammonium group in lysine.

In this way, all the amino groups of the lysine are deprotonated, except for the most external ones and they can be used for the binding/coupling. This type of conjugation comes with disadvantages. I) If the coupling happens on the CDRs of the Ab, the affinity of the ADC for the antigen can decrease. We obtain a heterogeneous mixture of positional isomers of the Ab. This can be seen with the DAR distribution. For this type of conjugate, it's large

(from 0 to 8). In order to narrow it, we can eliminate the first one (so DAR 0) because it's useless (no drug attached) and the last one (so DAR 8) because the number of drugs per Ab is too high (= increase in the general hydrophobicity that leads to aggregation and precipitation of the ADC).

- Site specific coupling. This type of conjugation is called site specific because we can select where the conjugation occurs. There are 2 different methods: enzymatic and not enzymatic.

  Not enzymatic method: 1) Reduction of natural cysteine, 2) Thiomab, 3) Insertion of not natural AA.

  1) It involves interchain disulphide bonds of cysteine of the Ab. This group must be "activated" previously: the disulphide bond must be reduced in order to free the single thiol group that can react with the linker. In this case the DAR distribution is less wide than the previous one and it's an advantage because it means more specificity. However, the reduction can decrease the activity of the ADC if it happens also on the intrachain disulphide bonds. These bonds are fundamental for the stability and for maintaining the structure of the Ab.

  2) Thiomab[14]: this approach consists of the insertion of a natural cysteine in specific sites of the Ab. Also for this, the reduction of the inserted disulphide bonds is necessary and it must be followed by a mild reoxidation of the original disulphide bonds, that can be reduced in the previous step. One of the advantages can be a low drug loading (generally a DAR 2 is obtained) to avoid aggregation and precipitation.

  3) The main advantage of inserting non-natural AA in the aminoacidic sequence through stop codons is the introduction of new and different active groups, that can be used for the conjugation. Two examples are para-acetyl phenylalanine (pAcPhe) and selenocysteine (Sec). For the first one, this AA contains a ketone group that can be conjugated to a linker containing an alkoxyamine group through an oxime bond. This insertion occurs with the substitution of a UAG stop codon in the desired position. The new AA is loaded into the corresponding tRNA through a muted tRNA synthetases. The tRNA is able to recognise the UAG codon

and consequently incorporate the new AA associated, without stopping the process.

The selenocysteine instead are characterize by the lack of a sulphur atom that is substituted with a selenium atom. The final molecule is a more reactive nucleophile, due to the presence of a selenate group instead of the thiolate group, and therefore the conjugation at electrophile molecules that activate the selenocysteine is possible. Normally, the UGA codon stops the transcription process. If there is a SECIS (Sec Insertion Sequence) in the 3' position of the UTR (UnTrascripted Region) of a protein, a new mRNA containing a selenocysteine in the UGA codon is formed. This process can be also engineered in a gene that doesn't contain a selenocysteine, by the insertion of a UGA codon and a SECIS sequence in the 3' position, in order to obtain a new Ab with one or more selenocysteines incorporated.

Enzymatic method[15]: 1) Transglutaminase and 2) Sortase A.

1) Transglutaminase recognises residues of glutamine. In a full Ab there are 2 residues of glutamine but they are in the Fc region so they cannot be used. The strategy consists of inserting glutamine residues that can be specifically recognised by transglutaminase.

2) Sortase A is a thiol-containing trans-peptidase. The substrate is the LPXTG motif (Leu-Pro-Any-Thr-Gly), that is contained in the recombinant protein. After cleavage of the LPXTG motif at the Gly level, Sortase forms a thioester intermediate. This intermediate can be attacked by a (Gly)n containing molecule, leading to C-terminus coupling.

1.4.5: ADC cell internalization

Once the ADC is injected in the bloodstream and arrived in the tumour microenvironment it has to release the drug in order to exploit its function. The release of the drug depends on the type of linker used[16].

There are two possible pathways: 1) target-directed and 2) target-enhanced.

1) Target-directed pathway: this pathway is common for liquid tumour. The circulating conjugate binds only to the cells that express the specific antigen. Then it's internalized in lysosomes and the acidic pH helps degrade the linker (if present) and release the drug.

2) Target-enhanced pathway: this pathway instead is common for solid tumour. The biggest problem for this type of tumour is the difficulty to penetrate the cancerous mass due to a very dense stroma. For this reason, it can be useful to release the drug in the extracellular matrix (local tumour environment). In this case, the conjugate doesn't have to be internalized in the tumour cells in order to release the drug. This is called 'bystander effect'. The advantage is that the drug can reach various cells simultaneously, but the disadvantage is that if the drug reaches the bloodstream, it can cause systemic toxicity. Also for this pathway, the specificity is obtained with the recognition of the antigen express on the surface of the carcinogenic cells.
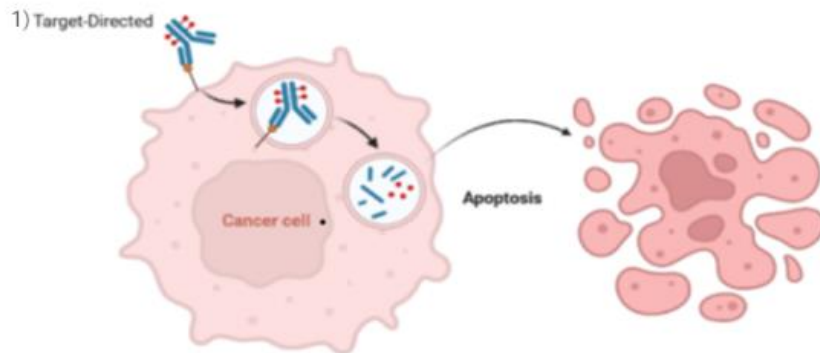
Figure 1.11: 1) Target-directed pathway. Imagine created with Biorender.com
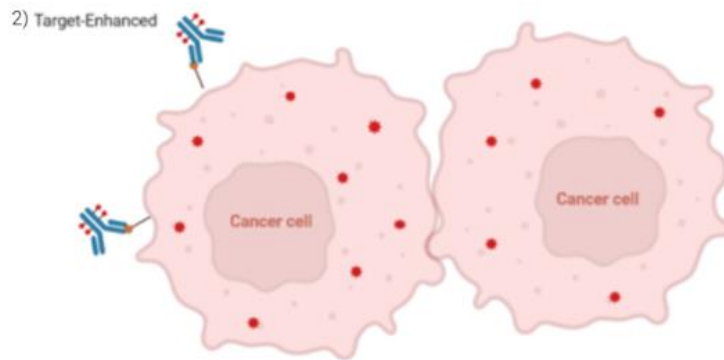
Figure 1.12: 2) Target-enhanced pathway. Imagine created with Biorender.com

1.4.6 Glypican 1

Glypican 1 is one of the 6 heparan sulphate proteoglycans (HSPGs) that are located on the cells surface[17]. These proteoglycans can be divided into 3 main classes, depending on the location: membrane HSPGs, secreted extracellular matrix HSPGs and secretory vesicle proteoglycans. I am interested in the membrane HSPGs.

They are glycoproteins composed by a protein core, 3 heparan sulphate glycosaminoglycan chains and a glycosyl-phosphatidylinositol anchor. This anchor allows the binding to the cell surface through the C-terminus of the proteoglycan.

In particular, GPC-1 contains 558 AA and it's composed of:

1. Secretory signal peptide (residues from 1 to 23)
2. N-terminal core protein (residues from 24 to 474)
3. Attachment region with HSPG chains (residues from 475 to 530)
4. GPI anchor (residues from 531 to 558)
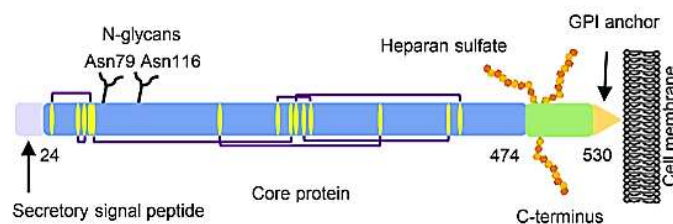
Figure 1.13: GPC-1 structure.

GPC-1 is usually overexpressed in different types of cancer, such as breast cancer, oesophageal squamous cell carcinoma, glioma, pancreatic cancer. It can be used as a therapeutic agent/target.

It acts as a co-receptor for lots of signalling molecules that allow the activation or deactivation of specific pathways. As it is shown in the image below, the most common are fibroblast growth factors (FGFs), vascular endothelial growth factor-A (VEGF-A), transforming growth factor b (TGF-B). They all regulate cell growth and differentiation.
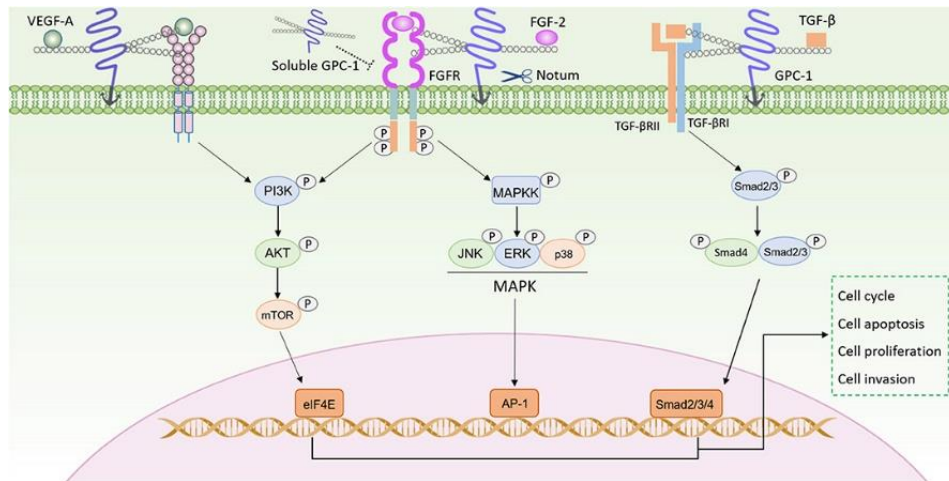
Figure 1.14: GPC-1 mediated pathways

GPC-1 can also be used as a biomarker for tumour diagnosis. GPC-1 is present in two different forms: one bounded to the membrane (insoluble) and the other one secreted (soluble). If the GPC-1 is cleaved, it can be released in the bloodstream. High level of GPC-1 (secreted) in the serum is connected to cancer. In fact, higher level of GPC-1 was detected in patients affected by prostatic cancer, breast cancer and pancreatic cancer. However, for pancreatic cancer, the use of GPC-1 as a biomarker is not efficient because it's difficult to distinguish between malign and benign pancreatic cancer.

1.4.7 Pancreatic tumour

Pancreatic cancer is one of the most aggressive types of cancer.

It can be divided into 2 main classes[18]: adenocarcinomas and non-adenocarcinomas. Adenocarcinomas generally start from the part of the pancreas that produces digestive enzymes. Non-adenocarcinomas are called like this because they are not caused by the digestive enzymes but by the hormone-producing pancreatic cells. They are neuroendocrine tumours, and they are less aggressive than adenocarcinomas.

The main symptoms are abdominal or back pain, yellow skin, dark urine, weight loss and loss of appetite[19]. These symptoms are not so alarming so when the diagnosis is done, generally the tumour is metastasized.

The diagnosis can be done by ultrasound or computed tomography, blood tests and biopsy.

The possible treatments that can be done are radiotherapy, chemotherapy, palliative care or a combination of them, depending on the cancer stage. The only treatment that can cure adenocarcinomas is surgery[20].

Also pancreatic carcer cells are characterized by an increase in the expression of specific factor, that acts as pro-angiogenic factors. In particular, pancreatic ductal adenocarcinoma cells are characterized by a high expression of GPC1[21].
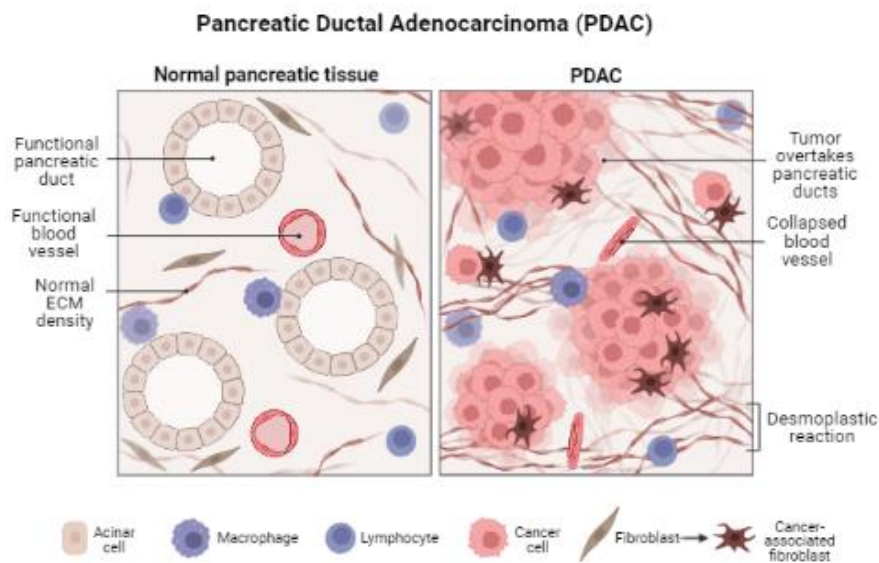
Figure 1.15: PDAC anatomy

2.0 MATERIALS AND METHODS

Solvents used: EtOH, MeOH, ACN, DMSO, TFA, 2-Propanol, Acetic Acid from Sigma-Aldrich Co. (St. Louis, USA).

Salts used: ammonium sulphate, sodium chloride, monobasic sodium phosphate, potassium chloride, EDTA, HEPES were bought from Sigma-Aldrich Co. (St Louis, USA) and Boric Acid from Prolabo (Paris, France).

IgM produced by CRO (Centro di Riferimento Oncologico), Aviano.
BSA (Bovine Serum Albumin) from Sigma-Aldrich Co. (St Louis, USA) and Trastuzumab from Farmacia Vaticana.

Linkers: mc-Val-Cit-PAB-MMAE (Cleavable linker) and SuO-Val-Cit-PAB-MMAE (Cleavable linker) from MedChemExpress, USA.

Reducing agents: DTT and TCEP were bought from Sigma-Aldrich Co. (St Louis, USA).

For the determination of the protein concentration, I used the BCA Protein Assay Kit, bought from Sigma-Aldrich Co. (St Louis, USA) with Corning® 96 Well TC-Treated Microplates from Merck, Darmstadt, Germany.
In order to read the microplates, I used the Microplate Autoreader EL311SX (Vinooski, VT, USA).

For SDS-PAGE: Mini-PROTEAN TGX Gels from Bio-Rad Laboratories, USA; the samples were prepared using Loading Buffer 2X Laemmli Sample Buffer (TRIS/Glycine/SDS 10X) and the Running Buffer was prepared from 10 X TGS Running Buffer, both from Bio-Rad Laboratories, USA; Precision Plus Protein Dual Color Standards from Bio-Rad Laboratories, USA; Coomassie Brilliant Blue R-250 from Bio-Rad Laboratories, USA.
For the cytotoxicity evaluation, a CellTiter from Promega was used.

Methods

Proteins were dialyzed with Slide-A-Lyzer^{TM} Dialysis Cassettes (MWCO 3.5 kDa) from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Buffers and solutions were filtrated using Millipore filter made of cellulose acetate with porosity of 0.22 μm (Benford, USA) and sterile Minisart syringe filters with porosity of 0.22 μm da from Sartorius Stedim Lab. (Stonehouse, UK).

Scales used: Gibertini mod. E50S/2 Semi-Micro Balance 0.01 mg (Milano, Italy) and Sartorius mod. Entris 2201-1S (Goettingen, Germany).

To measure the pH: pH-meter model 82 Radiometer (Copenhagen, Danimarca) with Metrohm 794 Basic Tritino electrode (Herisau, Svizzera).

To sonicate the solvents and buffers: Branson-Emerson 5210 Ultrasonic Cleaner (Danbury, CT, USA).

UV-VIS measure: UV-VIS spectrophotometer Evolution 201 from Thermo Scientific (Waltham, Massachusetts, USA).

To concentrate the sample: 50 kDa or 100 kDa Amicon in PES (Merck, Darmstadt, Germany) and with a Hettich Zentrifugen mod MIRKO 200 Centrifuge.

Thermomixers from Eppendorf ThermoMixer C from Merck, Darmstadt, Germany and Eppendorf Scilogex D3024.

For the purification of the conjugates an AKTA Purifier FPLC (GE Healthcare, Uppsala, Sweden) coupled with a Superose 12/10300 GL (GE Healthcare, Uppsala, Sweden) was used. The conjugates were characterized by using an Agilent 1260 Infinity HPLC (Agilent Technologies, Santa Clara, USA) coupled with BioBasic™ 4 (diameter 1 mm, length 50 mm, surface area 100 m^2/g, pore size 300 Å, particle size 5 μm) from ThermoFisher Scientific, USA for RP-HPLC.

2.1 IgM characterization

The characterization of any protein can be exploited by different techniques, such as:

1. Ultraviolet-Visible spectroscopy -> UV measure
2. Chromatography -> Size Exclusion Chromatography
3. Electrophoresis -> SDS-PAGE
4. Colorimetric technique -> Bicinchoninic acid assay

1. UV measure: the measure is done with a spectrophotometer. I can obtain the concentration of the sample, applying the Lambert Beer Law.

$$A = \varepsilon * b * c$$

Where: A = Absorbance of the sample,

ε = Specific extinction coefficient (mL*mg^{-1}*cm^{-1}),

b = Optical path length (cm),

c = Concentration of the sample (mg/mL).

The cuvette length is 1 cm so b = 1 cm. Knowing this, the equation becomes:

$$c = A / \varepsilon.$$

It's important to find the correct and precise concentration of the initial rate of the IgM because I need to know the exact mg of protein that I have.

$$c = mg/mL \text{ -> } mg = c * mL$$

For the characterization of this IgM, I used 1.32 mL*mg^{-1}*cm^{-1} as ε value.

2. SEC: This chromatographic technique allows the separation of a solution based on the size of the molecules that are present in the solution[22].

Bigger molecules will elute before than smaller molecules because they are characterized by a higher hydrodynamic volume. As a consequence, bigger molecules will enter in the resin's pores in the same way of the smaller molecules but the retention in the pores is lower and so they are eluted before. Bigger aggregates have a lower retention time than smaller aggregates. The column I used was a Superose 12 10/300 and it's composed by highly cross-linked agarose polymer. It separates from 1000 Da to 300000 Da and the pores have a dimension of 11 μm. Before performing any analysis, the column should be equilibrated in the buffer in which I want my final compound.

3. SDS-PAGE: This technique allows to separate and determine the apparent molecular weight of proteins[23]. The separation takes place thanks to a differential migration of charged species in an electric field. SDS is an anionic detergent that binds to the hydrophobic surface of the proteins. As a result of this binding, there is a partial denaturation of the protein and the acquisition of a negative charge every 2 AA. In this way, every protein will have a negative charge and they will migrate following the electric flow.

The electrophoretic run of the proteins depends on their ratio charge / hydrodynamic radius. Since there are proteins characterized by different charges and hydrodynamic radius but with the same ratio, they cannot be separated, and they co-migrate. The addition of the SDS is fundamental in order to maintain this ratio constant for every protein and so the separation can occur only thanks to the hydrodynamic radius value.

Every protein will have a number of negative charge equal to the number of SDS molecules attached: bigger proteins will have a higher number of negative charge than smaller proteins so proteins with a higher molecular weight will migrate less than proteins with a lower molecular weight.

The gel used is a precast Mini-PROTEAN® TGX™ with a polyacrylamide gradient of 4-15%. This gel also contains 0.02% of NaN3, used as antimicrobial agent. The running buffer is composed by 10% of 10X Tris/Glycine Buffer so

lution, make to volume with milliQ water.

Sample preparation:

The amount of protein that I injected in the wells of the gel is 10 μg. The volume obtained is diluted with the same volume of non-reducing or reducing (with 2-mercaptoethanol) Gel Loading Buffer (1:1 ratio).

The sample GLB is composed by the buffer (Tris-Glycine), glycerol (to increase the viscosity, in order to reduce the diffusion of the sample in the well), Bromophenol blue (a tracer for the electrophoretic run), SDS and 2-mercaptoethanol (for the reduction protocol).

After adding the GLB, the sample must be kept under stirring and at 100 °C in the thermomixer for 3 minutes. After this, the sample can be injected in the gel and the run can proceed at 60 mA and 250 V, for 45 minutes.

Right after the run, the gel is kept for 10 minutes in a solution made of 20 mL of milliQ water and 170 μL of perchloric acid.

Gel staining:

In order to see the protein separation, the gel need to be stained. The staining is done with a solution of 0.025 % p/v of Coomassie Brilliant Blue R-250, 40 % of Methanol, 7 % acetic acid in milliQ water.

The gel is kept in this solution for 1 or 2 hours, until the gel reaches a blue staining.

Gel destaining:

The destaining solution is done with 40 % of methanol, 7 % of glacial acetic acid in milliQ water. The gel is kept in this solution until the aspecific staining disappears. At the end, the only bands visible will be the ones of the standards and the proteins.

Storage:

After the destaining procedure, the gel is rinsed and kept in milliQ water.

4. BCA: Bicinchoninic acid assay.

This colorimetric assay, also known as the Smith assay[24] after its inventor Paul K. Smith, is used to quantify and determine the concentration of the protein sample, here the IgM conjugated with MMAE. An UV measure at the spectrophotometer cannot be done in order to determine the protein concentration, applying the Lambert Beer Law, because also MMAE absorbs at 280 nm. The protein concentration is related to the colour intensity of the final solution. It's a technique that requires heating for colour development (37°C for 30 minutes at 300 rpm, after the working reagent addition).

At the beginning of the assay, the colour solution is green but if in the solution there is a protein, the colour will become purple after heating. The first reaction that occur is the reduction of Cu^{2+} of $CuSO_4$ (that gives the green colour) to Cu^{1+} by proteins in a basic environment. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution.

$$\text{PROTEIN} \quad + \quad Cu^{2+} \quad \xrightarrow{OH^-} \quad Cu^{1+}$$

PROTEIN   CUPROUS ION   CUPRIC ION
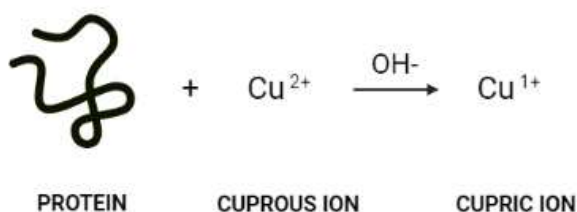
Figure 2.1: Reaction between protein and Cuprous ion of $CuSO_4$.

The second reaction is the binding of 2 molecules of bicinchoninic acid (BCA) to every Cu^{1+} atom, forming a purple complex that absorbs at 562 nm in the UV spectrum. This assay is influenced by the presence of specific amino acids residues, such as cysteine, cystine, tyrosine and tryptophan in the protein.
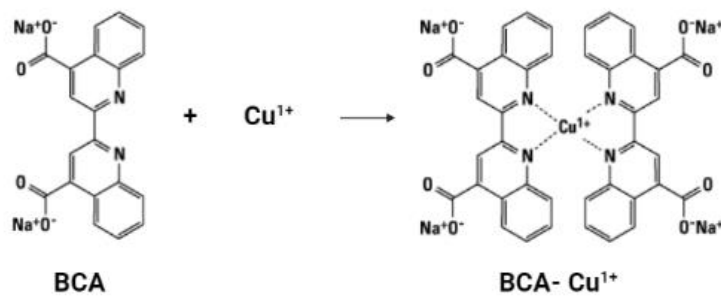
Figure 2.2: Reaction between BCA and Cupric ion (Cu^{1+}) formed in the first reaction to form a coloured complex. Imagine created with Biorender.com

For this assay, a Corning® 96 Well TC-Treated Microplates is used. Generally, for the BCA assay, in addition to the protein samples, a calibration line and blanks of the solvents used are added as reference, in order to interpret the results obtained at the spectrophotometer. For the calibration line, I used BSA (Bovine Serum Albumin) at increasing concentration (from 0.1 mg/ml to 1 mg/ml), diluted with milliQ water. For blanks, I used milliQ water and PBS 1X buffer pH 6.5.

The reactive solution is composed by 2 stable reagents that combined together make the working reagent, $CuSO_4$ and bicinchoninic acid solution. The bicinchoninic acid solution, also called Solution A, is composed of bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 N NaOH (Final pH 11.25). The $CuSO_4$ solution, also called Solution B, contains 4% (w/v) of Copper (II) sulphate pentahydrate. These 2 reagents are mixed in 1:50 ratio, where 1 part is for Solution B and 50 parts for Solution A. The working reagent will have a light blue/light green colour. The working agent should be prepared immediately before adding it to the samples.

The sample is added in 1:8 ratio compared to the final volume of the working reagent, where 1 part is for the sample and 8 parts is for the working reagent.

Samples	µL samples	Working reagent
Water	25 µL	200 µL
PSB 1X pH 6.5	25 µL	200 µL
BSA calibration line (From 0.1 mg/ml to 1 mg/ml)	25 µL	200 µL
Protein samples	25 µL	200 µL

Table 2.1: Summary table for the BCA assay preparation.

2.2 Cleavable linkers structure

I used 2 different types of cleavable linkers: mc-Val-Cit-PAB-MMAE and SuO-Val-Cit-PAB-MMAE. They are both cleavable by proteolytic enzymes, such as Cathepsin B, thanks to the Valine-Citrulline dipeptide.

They are characterized by specific units:

- Reactive group -> mc (MaleimidoCaproyl group) or SuO (N-hydroxy succinimidyl ester group)
- Dipeptide -> Valine-Citrulline (for both)
- Spacer -> PAB (Para AminoBenzyloxycarbonyl group) (for both)
- Cytotoxic drug -> MMAE (for both)

What is important to underline is the reactive group. This group allows the attachment to the antibody. For this reason, the linkers should be bifunctional in order to conjugate both the antibody and the cytotoxic drug.

For MC (MaleimidoCaproyl) based linker, the binding with the Ab occurs through the interchain disulphide bond of Cysteine (previously reduced to free thiols). In particular, the maleimide group of the linker binds to the thiol group of cysteine of the Ab, forming a thiosuccinimide, through a Michael reaction.

For SuO (N-hydroxy succinimidyl ester) based linker instead, the binding occurs between the amino groups of N-terminal amino acids, specifically the ε amino groups of lysine , of the Ab and activated carboxylic group of the reactive group of the linker to form a stable amide, with the release of N-hydroxy succinimide.

The biggest difference between these 2 linkers is the types of conjugation. With the MC linker we can obtain a directed or site-specific conjugation because it can occur only on interchain cysteines. Instead with the SuO linker the conjugation is random: it can happen on every amino group of the N-terminal amino acids of the Ab (but the conjugation reaction is set up to conjugate only on the ε amino groups of lysine , the most exposed ones to the solvents).

Another difference is the fact that MC linker products are less stable *in vivo* than the SuO linker products because of the possible elimination of the thiosuccinimide through a retro-Michael reaction or by thiol exchange.

Figure 2.3: Schematic representation of A. Lysine Conjugate and B. Cysteine Conjugate, both with an IgG. Imagine created with Biorender.com

2.2.1 mc-Val-Cit-PAB-MMAE

In this thesis project, the linker-drug moiety mc-Val-Cit-PAB-MMAE is used to realize cysteine conjugate. The reaction occurs between the maleimide group of the linker and the thiol group of the antibody. Cysteine conjugation generally occurs on interchain disulphide bonds because they are more accessible to solvents than intrachain ones. Intrachain conjugation is also riskier because it can compromise the structure and consequently the activity of the Ab. This linker generates a stable covalent bond with the Ab, that can be cleaved by specific proteases.
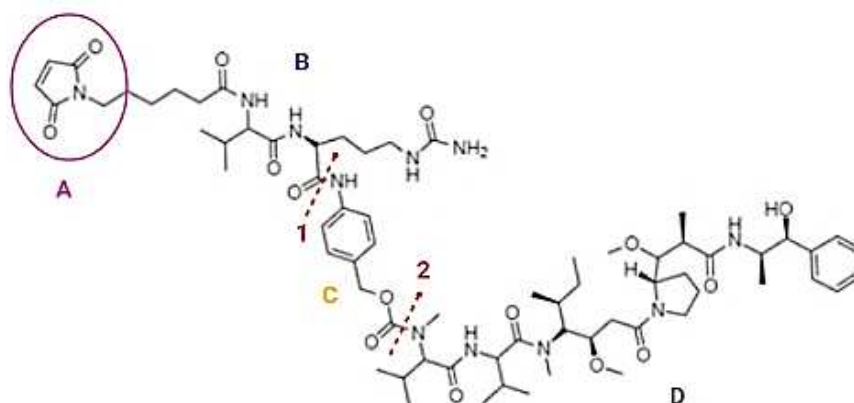
Figure 2.4: Linker structure.

A: Maleimide: reactive group toward thiol groups of the antibody

B: Val-Cit: Valine-citrulline dipeptide

C: PAB (Para AminoBenzyloxycarbonyl group)

D: MMAE

1: Protease cleavage site between Val-Cit dipeptide and PAB

2: Amidic bond between PAB and MMAE
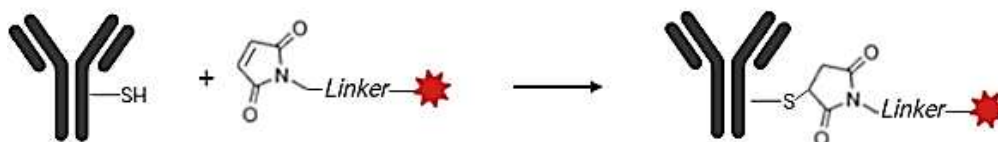
Figure 2.5: Reaction between free SH of the antibody and maleimide group of the linker, through a Michael reaction.

2.2.2 SuO-Val-Cit-PAB-MMAE

This linker is used to realize lysine conjugate. The reaction occurs between the N hydroxyl succinyl ester group of the linker and the lysine group of the antibody, forming a stable amide.

The biggest disadvantage of lysine conjugation is the random conjugation. The binding can occur on every available amino group, α-amino group of N-terminal amino acids and ε-amino group of the exposed lysines. In order to direct the binding only on ε-amino group, the pH of the reaction can be set around 8. In this

way, only external amino group will be protonated and so more nucleophilic than usual.
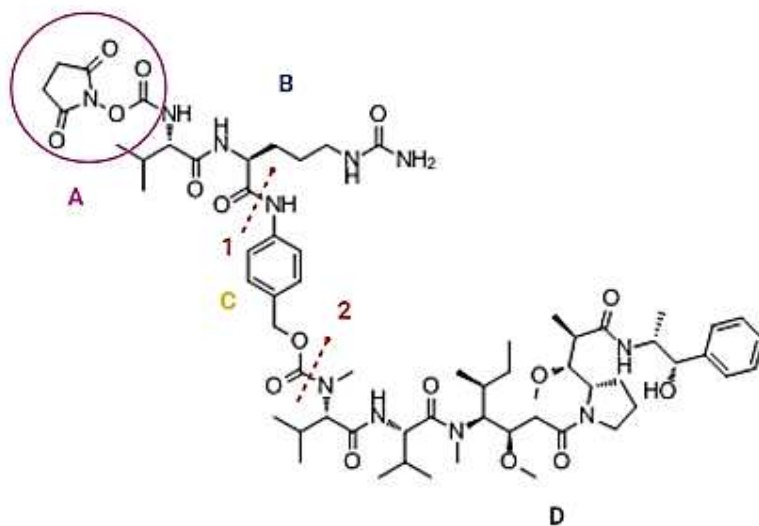
Figure 2.6: Linker structure.

      A: SuO: N hydroxy succinimidyl ester group

      B: Val-Cit: Valine-citrulline dipeptide

      C: PAB (Para AminoBenzyloxycarbonyl group)

      D: MMAE

      1: Protease cleavage site between Val-Cit dipeptide and PAB

      2: Amidic bond between PAB and MMAE
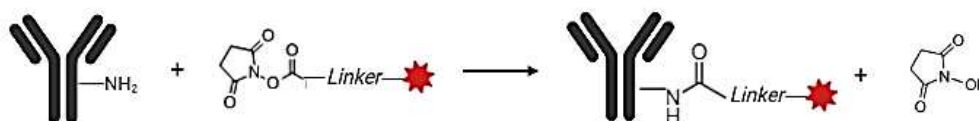
Figure 2.7: Reaction between Ɛ-amino group of the Ab and N-hydroxy Succinimidyl ester group of the linker, with the elimination of the N-hydroxy Succinimide derivate.
Imagine created with Biorender.com

2.3 Buffers preparation

Buffers for the double step synthesis:

| PBS 10 X	This buffer is composed by 80.06 g of NaCl (MW: 58.44 g/mol), 2.01 g of KCl (MW: 74.55 g/mol), 14.2 g of Na_2HPO_4 (MW: 119.8 g/mol) and 2.72 g of KH_2PO_4 (MW: 136.09 g/mol). The pH of this buffer is set at 6.5.
Borate buffer	This buffer is used to exchange the buffer in which the IgM is. It's composed of 25 mM NaCl (MW: 58.44 g/mol), 25 mM Boric Acid (MW: 61.83 g/mol) and 2 mM EDTA disodium salt (MW: 372.24 g/mol). The final pH of the solution must be 8.
PBS pH 7.4	This buffer is used in the reducing step. It's composed of PBS 10 X pH 6.5 diluted 1:10 and 2 mM EDTA disodium salt (MW: 372.24 g/mol). The final pH of the solution must be 7.4.
PBS pH 6.5	This buffer is used in the final purification step. It's composed of PBS 10 X pH 6.5 diluted 1:10.

Buffers for the one step synthesis:

PBS 10 X	This buffer is composed by 80.06 g of NaCl (MW: 58.44 g/mol), 2.01 g of KCl (MW: 74.55 g/mol), 14.2 g of Na_2HPO_4 (MW: 119.8 g/mol) and 2.72 g of KH_2PO_4 (MW: 136.09 g/mol). The pH of this buffer is set at 6.5.
HEPES buffer	This buffer is used to exchange the buffer in which the IgM is and for the conjugation step. It's composed of 0.1 M of HEPES (MW: 238.30 g/mol). The final pH of the solution must be 8.
PBS 1 X buffer	Dilution 1:10 of PBS 10X.
PBS pH 6.5	This buffer is used in the final purification step. It's composed of PBS 10 X pH 6.5 diluted 1:10.

2.3 Double step synthesis

mc-VC-PAB- MMAE
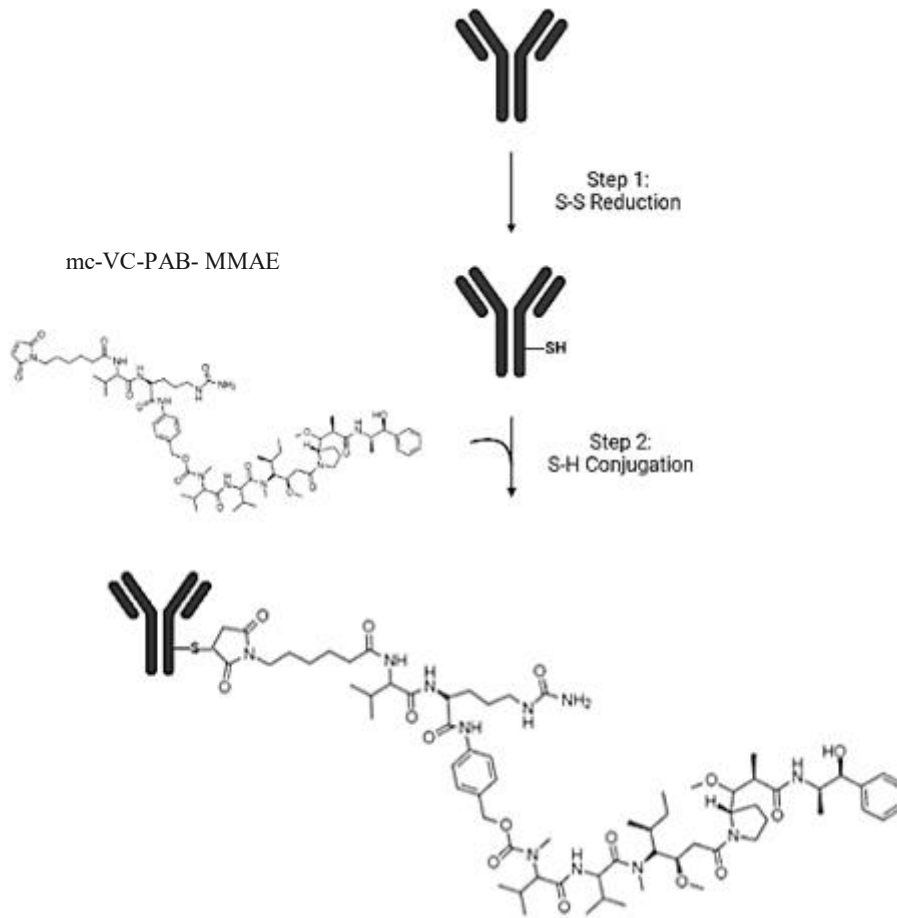
Figure 1.8: Double step synthesis. Imagine created with ChemDraw and Biorender.com

This type of synthesis is used to obtain cysteine conjugates[26]. The aim is to conjugate to interchain cysteine. In order to do that, the reduction of interchain disulphide bonds is necessary. The most used reducing agents are DTT and TCEP. Before the reduction step, the IgM rate needs to be dialyzed against Borate buffer and then concentrated up to 4 mg/ml (more or less) with a 50 kDa Amicon. This concentration step is necessary because the initial concentration of the IgM is lower than the concentration needed for the reduction step reaction.

After concentration, the reduction reaction can be set. The IgM concentration is equal to 2.5 mg/ml instead the TCEP concentration is variable.

The next step is purification at the FPLC instrument with Superose 12 10/300 column, used for SEC. This step is carried out in order to remove the excess of TCEP in the reaction and to exchange buffer (from Borate Buffer to PBS pH 7.4 + EDTA). EDTA is used as chelating agent and helps preventing the reoxidation of the sulfhydryl groups by divalent metals such as Zn^{2+}, Cu^{2+} and Mg^{2+}. It's also important for the inactivation of proteases that can inactivate the antibody.

After purification, the IgM reduced is diluted so it has to be concentrated again about 10 times with a 50 kDa Amicon.

At this point, the IgM can be conjugated. The IgM concentration for the conjugation reaction is set to 2.5 mg/ml. The aim of the conjugation is 4 molecules of MMAE per monomers (DAR = 20).

Also after conjugation, a purification step at the FPLC instrument with Superose 12 10/300 column is required. The purification is important to eliminate the excess of Linker-Drug used. The reaction buffer changes from PSB pH 7.4 + EDTA to PBS pH 6.5.

A BCA assay can be performed to determine the concentration (and consequently the amount) of the final conjugate produced.

2.3.2 Disulphide bonds reduction optimization

The reduction step is the limiting factor of the conjugation reaction because if the reduction doesn't happen or is not complete, the conjugation cannot be controlled. It's important to test different reduction conditions, to find the one that allows the reduction of only interchain disulphide bonds. If the reduction occurs also on the intrachain disulphide bonds, the result will be the destruction of the Ab that will lead to denaturation and inactivation.

The reducing agent used are DTT and TCEP at different molarity.

The reduction with TCEP is set at 2.5 mg/ml and in Borate buffer pH 8.

The reduction with DTT is set at 1 mg/ml and in TRIS HCl 50 mM buffer pH 8.

RX agent	mM	mmoles RX agent	mmoles IgM	Eq RX agent	Result RX
DTT	50 mM	$5*10^{-3}$	$3*10^{-8}$	$1.67*10^5$	Total
TCEP	0.2 mM	$1.56*10^{-6}$	$5.21*10^{-7}$	3	No
TCEP	1 mM	$2.8*10^{-4}$	$7.29*10^{-7}$	384	Partial
TCEP	3 Mm	$6*10^{-4}$	$5.21*10^{-7}$	1151.6	IgM fragm.
TCEP	10 mM	$2*10^{-3}$	$5.21*10^{-7}$	3839	IgM fragm.

Table 2.2: Reduction test summary

At the concentration used, DTT leads to total reduction of the disulphide bond, interchain and intrachain ones.

After every reduction reaction, an SDS-PAGE and a purification in SEC were performed. The SDS-PAGE was performed in non-reducing condition (without β-mercaptoethanol), to evaluate the reducing agent efficacy. STD (Dual Color standard) and IgM pure (after the first concentration step) were used as a comparison.

The purification instead was always performed in FPLC with a SEC column in order to remove the excess of reducing agent used.

2.3.3 Reduction comparison between IgM and IgG

In order to understand the reduction behaviour of TCEP, I reduced an IgG (in this case I used TRS, at 5.85 mg/mL, with the same equivalents used for the IgM. The aim is to find similarities in the reduction pattern of the IgM and the IgG.

In the table 2.3, there is a summary of the reduction with TCEP as reducing agent, showing the correspondence of the equivalents and concentration of TCEP for IgM and IgG.

The reduction reaction of the IgG in both conditions is set at 2.5 mg/ml.

In order to determine the equivalents of TCEP, the equivalents of the IgM must be calculated. Knowing the mM of TCEP in the reaction and the mmoles of the IgM

and TCEP, I can obtain the equivalents of TCEP (the reference is the equivalents of the IgM: 1 Equivalent).

I considered TCEP 3 mM, corresponding to 1152 Equivalents, and TCEP 10 mM, corresponding to 3839 Equivalents.

For the reduction with 1152 Equivalents of TCEP, I used 51.2 µL of IgG (at 5.85 mg/ml) in order to have 0.3 mg of IgG in the reduction reaction. I added 18.8 µL of Borate Buffer, directly in the Eppendorf where I put the IgG. Then I added 50 µL of TCEP 20 mM under stirring and then I pushed nitrogen into the Eppendorf to avoid oxidation. The reaction is kept at 37°C in a thermomixer for 1 hour.

For the reduction with 3839 Equivalents of TCEP, I used 51.2 µL of IgG (at 5.85 mg/ml) in order to have 0.3 mg of IgG I the reduction reaction. I added 8.8 µL of Borate Buffer and 60 µL of TCEP 66.6 mM under stirring in the same Eppendorf. Also for this reaction, nitrogen is added into the Eppendorf and then is kept at 37 °C for 1 hour.

An SDS-PAGE was made with every sample to evaluate the reduction pattern.

Antibody	TCEP Equivalents	TCEP Concentration
IgM	1152 Eq	3 Mm
IgM	3839 Eq	10 mM
IgG	1152 Eq	20 mM
IgG	3839 Eq	66.6 mM

Table 2.3: Summary of the condition of the reduction with TCEP, on IgG and IgM.

2.3.4 1° Attempt: TCEP 7.814 µM + Linker - Drug 6 µM

0.5 mg of IgM is dialyzed against Borate Buffer. After dialysis, the IgM is collected and concentrated up to 4.19 mg/ml with a 50 kDa Amicon. I used 119.3 µL of IgM (at 4.19 mg/ml) in order to have 0.5 mg of IgM in the reduction reaction (at 2.5 mg/ml). I added 72.9 µL of Borate Buffer, directly in the Eppendorf where I put the IgM. Then I added 7.8 µL of TCEP 200 µM (to have TCEP $2.239 * 10^{-3}$ mg/ml in reaction) under stirring and then I pushed nitrogen into the Eppendorf to

avoid oxidation. I started using 3 Equivalents of reducing agent to test the reduction. The reaction is kept at 37°C in a thermomixer for 1 hour. It's important not to leave the reaction going longer than 1 hour because otherwise the reduction can continue. In order to stop the reaction, the Eppendorf is put in ice.

	Eq	MW	Conc. , mg/mL	Mmoles	Mg	µL
IgM	1	960000	2.5	$5.21*10^{-7}$	0.5	119.3
TCEP	3	286.65	$2.239*10^{-3}$	$1.56*10^{-6}$	$4.48*10^{-4}$	7.8
BORATE						72.9

Table 2.4: Reduction step with TCEP 7.814 µM

After the reduction, the reaction is purified at the FPLC instrument with Superose 12 10/300 column previously equilibrated in PBS pH 7.4 + 2mM EDTA, used for SEC.

Through a UV measure at the spectrophotometer, I can estimate the concentration of the purified IgM. The wavelengths that I set in the spectrophotometer are: 280 nm, 350 nm and 248 nm. 280 nm is the wavelength of maximum absorption in the near-UV of the chromophore of proteins (aromatic ring). 350 nm is the wavelength of maximum absorption in the near-UV of aggregates and the scattering of the solution (it can also be particulate present in the solution). 248 nm is the wavelength of absorption in the near-UV of the cytotoxic drug MMAE.

The concentration of the IgM purified after concentration in 50 KDa Amicon is 1.53 mg/ml. For the conjugation reaction, I used 232 µL of the IgM purified (at 1.53 mg/ml) in order to have 0.35 mg of IgM in the reaction (at 1 mg/ml) and then I added 87.5 µL of PBS pH 7.4 + 2 mM EDTA in the same Eppendorf. In another Eppendorf I put 5.8 µL of mc-VC-PAB-MMAE Linker-Drug (from dilution 1:100 of a solution at 50 mg/ml) and 29.7 µL of DMSO (the volume of DMSO used should be at least 10% of the final volume of the reaction) under continue stirring. The final step is adding the solution of (Linker-Drug + DMSO) directly in the solution of (IgM + PBS) always under stirring. The reaction is kept at 25°C in a thermomixer for 30 minutes.

	Eq	MW	Conc. , mg/mL	Mmoles	Mg	μL
IgM	1	960000	1	$3.70*10^{-7}$	0.354	232
PBS pH 7.4	/	/	/	/	/	87.5
Linker	6	1316.6	50	$2.22*10^{-6}$	0.0029	5.8
DMSO	/	/	/	/	/	29.7

Table 2.5: Conjugation step with 6 Eq of Linker.

The last step of the synthesis is the purification at the FPLC instrument with Superose 12 10/300 column equilibrated in PBS 1X pH 6.5, used for SEC. In this case, the aim is to remove the excess of unreacted linker to obtain a purer final product.

The BCA assay was performed using 6 blanks (3 blanks with milliQ water and 3 blanks with PBS 1X pH 6.5 buffer), 2 BSA calibration lines (from 0.1 mg/ml to 1 mg/ml, with milliQ water) and 3 IgM conjugated samples (in PBS 1X pH 6,5). The final concentration of the IgM conjugated was 0.2354 mg/ml (final volume: 980 μL).

2.3.5 2° Attempt: TCEP 1 mM + Linker - Drug 65.28 μM

0.7 mg of IgM is dialyzed against Borate Buffer. After dialysis, the IgM is collected and concentrated up to 4.66 mg/ml with a 50 kDa Amicon. I used 150 μL of IgM (at 4.66 mg/ml) in order to have 0.7 mg of IgM in the reduction reaction (at 2.5 mg/ml). I added 102 μL of Borate Buffer, directly in the Eppendorf where I put the IgM. Then I added 28 μL of TCEP 10 mM to have TCEP 1mM (in reaction the concentration of TCEP is 0.287 mg/ml) under stirring and then I pushed nitrogen into the Eppendorf in order to avoid air oxidation. The reaction is kept at 37°C in a thermomixer for 1 hour. After 1 hour, the Eppendorf is put in/on ice to stop the reaction.

	Eq	MW	Conc. , mg/mL	Mmoles	Mg	μL
IgM	1	960000	2.5	$7.29*10^{-7}$	0.7	150
TCEP	384	286.65	0.287	$2.8*10^{-4}$	0.0803	28
BORATE						102

Table 2.6: Reduction step with TCEP 1 mM.

After the reduction, the reaction is purified at the FPLC instrument with Superose 12 10/300 column equilibrated in PBS pH 7.4 + 2mM EDTA, used for SEC. After purification, the IgM reduced is diluted and it has to be concentrated in a 50 kDa Amicon. The concentration of the IgM purified after concentration is 3.1 mg/ml. For the conjugation reaction, I used 110 μL of the IgM purified (at 3.1 mg/ml) in order to have 0.341 mg of IgM in the reaction (at 2.5 mg/ml) and then I added 12.4 μL of PBS pH 7.4 + 2 Mm EDTA in the same Eppendorf. In another Eppendorf I put 2.34 μL of mc-VC-PAB-MMAE Linker-Drug (from dilution 1:100 of a solution at 50 mg/ml) and 11.26 μL of DMSO (the volume of DMSO used should be at least 10% of the final volume of the reaction) under continue stirring. The final step is adding the solution of (Linker-Drug + DMSO) directly in the solution of (IgM + PBS) always under stirring. For this reaction, I used an excess of 1.25 of linker (4 molecules of linker per monomer * 1.25 = 25 Eq) in order to guarantee the conjugation. The reaction is kept at 25°C in a thermomixer for 30 minutes.

	Eq	MW	Conc. , mg/mL	Mmoles	Mg	μL
IgM	1	960000	2.5	$3.55*10^{-7}$	0.341	110
PBS pH 7.4	/	/	/	/	/	12.4
Linker	25	1316.6	50	$8.88*10^{-6}$	0.01169	2.34
DMSO	/	/	/	/	/	11.26

Table 2.7: Conjugation step with 25 Eq of Linker.

The last step of the synthesis is the purification at the FPLC instrument with Superose 12 10/300 column equilibrated in PBS 1X pH 6.5, used for SEC. In this case, the aim is to remove the excess of unreacted linker to obtain a purer final product.

The BCA assay was performed using 6 blanks (3 blanks with milliQ water and 3 blanks with PBS 1X pH 6.5 buffer), 2 BSA calibration lines (from 0.1 mg/ml to 1 mg/ml, with milliQ water) and 3 IgM conjugated samples (in PBS 1X pH 6.5). The final concentration of the IgM conjugated was 0.177 mg/ml (final volume: 1160 μL).

2.4 One step synthesis

SuO-VC-Pab- MMAE

Elimination product:

N-hydroxy Succinimide
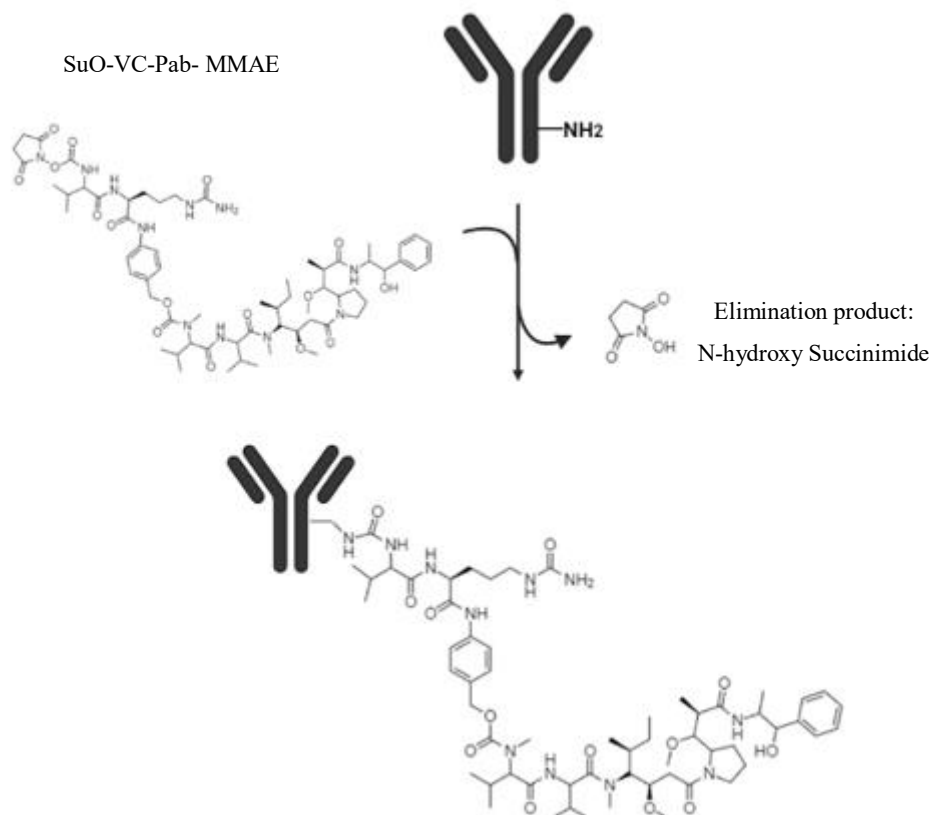
Figure 2.9: Single step synthesis. Imagine created with ChemDraw and Biorender.com

This type of synthesis is used to obtain lysine conjugates.

The first step of the reaction is the dialysis of the IgM rate against HEPES Buffer pH 8 and then concentration up to 4 mg/ml (more or less) with a 100 kDa Amicon. This concentration step is necessary because the initial concentration of the IgM is lower than the concentration required for the conjugation step reaction. After concentration, the conjugation reaction can be set. The IgM concentration is equal to 2.5 mg/ml instead the equivalents of linker are variable (the concentration of the linker is set at 4.6 mg/ml). The aim of the conjugation is 4 molecules of

MMAE per monomers (DAR = 20). The buffer used for the conjugation reaction is HEPES 0.1 M buffer at pH 8. The pH is fundamental in order to activate specific functional group, including the amino group.

The next step is purification at the FPLC instrument with Superose 12 10/300 column, used for SEC. This step is carried out in order to remove the excess of linker that hasn't reacted in the reaction and to exchange buffer (from HEPES 0.1 M buffer pH 8 to PBS 1X pH 6.5).

After purification, the IgM is diluted so it has to be concentrated again about 10 times with 100 kDa Amicon.

A BCA assay can be performed to determine the concentration (and consequently the amount) of the conjugate produced.

2.4.2 1° Attempt: Linker – Drug 102.8 µM (40 Eq of Linker – Drug, excess of Linker-Drug: 2 times)

0.5 mg of IgM is dialyzed against HEPES Buffer 0.1 M. After dialysis, the IgM is collected and concentrated up to 4.11 mg/ml with HEPES Buffer 0.1 M. I used 122 µL of IgM (at 4.11 mg/ml) in order to have 0.5 mg of IgM in the conjugation reaction (at 2.5 mg/ml). I added 58 µL of HEPES Buffer 0.1 M, directly in the Eppendorf where I put the IgM. In another Eppendorf I put 5.73 µL of SuO-VC-PAB-MMAE Linker-Drug (at 4.6 mg/ml) and 14.27 µL of DMSO (the volume of DMSO used should be at least 10% of the final volume of the reaction) under continue stirring. The final step is adding the solution of (Linker-Drug + DMSO) directly in the solution of (IgM + PBS) always under stirring. For this reaction, I used 40 equivalents of linker in order to guarantee the conjugation. The reaction is kept at 25°C in a thermomixer for 2 hours.

	Eq	MW	Conc. , mg/mL	Mmoles	Mg	μL
IgM	1	960000	2.5	$5.21*10^{-7}$	0.5	122
HEPES	/	/	/	/	/	58
Linker	40	1264.51	4.6	$2.084*10^{-5}$	0.026	5.73
DMSO	/	/	/	/	/	14.27

Table 2.8: Conjugation step with 40 Eq of Linker.

The last step of the synthesis is the purification at the FPLC instrument with Superose 12 10/300 column equilibrated in PBS 1 X pH 6.5, used for SEC. In this case, the aim is to remove the excess of unreacted linker to obtain a purer final product.

The BCA assay was performed using 6 blanks (3 blanks with milliQ water and 3 blanks with PBS 1X pH 6.5 buffer), 2 BSA calibration lines (from 0.1 mg/ml to 1 mg/ml, with milliQ water) and 3 IgM conjugated samples (in PBS 1X pH 6.5). The final concentration of the IgM conjugated was 0.7197 mg/ml (final volume: 322 μL).

2.4.3 2° Attempt: Linker – Drug 156.7 μM (60 Eq of Linker – Drug, excess of Linker-Drug: 3 times)

0.226 mg of IgM is dialyzed against HEPES Buffer 0.1 M. After dialysis, the IgM is collected and concentrated up to 4.11 mg/ml. I used 54.99 μL of IgM (at 4.11 mg/ml) in order to have 0.226 mg of IgM in the conjugation reaction (at 2.5 mg/ml). I added 26.37 μL of HEPES Buffer 0.1 M, directly in the Eppendorf where I put the IgM. In another Eppendorf I put 3.88 μL of SuO-VC-PAB-MMAE Linker-Drug (at 4.6 mg/ml) and 5.16 μL of DMSO (the volume of DMSO used should be at least 10% of the final volume of the reaction) under continue stirring. The final step is adding the solution of (Linker-Drug + DMSO) directly in the solution of (IgM + PBS) always under stirring. For this reaction, I used 60 equivalents of linker in order

to guarantee the conjugation. The reaction is kept at 25°C in a thermomixer for 2 hours.

	Eq	MW	Conc. , mg/mL	Mmoles	Mg	μL
IgM	1	960000	2.5	$2.354*10^{-7}$	0.226	54.9
HEPES	/	/	/	/	/	26.37
Linker	60	1264.51	4.6	$1.4125*10^{-5}$	0.0179	3.88
DMSO	/	/	/	/	/	5.16

Table 2.9: Conjugation reaction with 60 Eq of Linker.

The last step of the synthesis is the purification at the FPLC instrument with Superose 12 10/300 column equilibrated in PBS 1X pH 6.5, used for SEC. In this case, the aim is to remove the excess of unreacted linker to obtain a purer final product.

The BCA assay was performed using 6 blanks (3 blanks with milliQ water and 3 blanks with PBS 1X pH 6.5 buffer), 2 BSA calibration lines (from 0.1 mg/ml to 1 mg/ml with milliQ water) and 3 IgM conjugated samples (in PBS 1X pH 6.5). The final concentration of the IgM conjugated was 0.1517 mg/ml (final volume: 59 μL).

2.4.4 3° Attempt: Linker – Drug 181.9 μM (70 Eq of Linker – Drug, excess of Linker-Drug: 3.5 times)

2 mg of IgM is exchanged against HEPES Buffer 0.1 M. After that, the IgM is collected and concentrated up to 3.672 mg/ml. I used 544.66 μL of IgM (at 3.672 mg/ml) in order to have 2 mg of IgM in the conjugation reaction (at 2.5 mg/ml). I added 175.24 μL of HEPES Buffer 0.1 M, directly in the Eppendorf where I put the IgM. In another Eppendorf I put 40.1 μL of SuO-VC-PAB-MMAE Linker-Drug (at 4.6 mg/ml) and 40 μL of DMSO (the volume of DMSO used should be at least 10% of the final volume of the reaction) under continue stirring. The final step is adding

the solution of (Linker-Drug + DMSO) directly in the solution of (IgM + PBS) always under stirring. For this reaction, I used 70 equivalents of linker in order to guarantee the conjugation. The reaction is kept at 25°C in a thermomixer overnight.

	Eq	MW	Conc. , mg/mL	Mmoles	Mg	μL
IgM	1	960000	2.5	$2.083*10^{-6}$	2	544.66
HEPES	/	/	/	/	/	175.24
Linker	70	1264.51	4.6	$1.4581*10^{-4}$	0.184	40.1
DMSO	/	/	/	/	/	40

Table 2.10: Conjugation reaction with 70 Eq of Linker.

The last step of the synthesis is the purification at the FPLC instrument with Superose 12 10/300 column equilibrated in PBS 1X, used for SEC. In this case, the aim is to remove the excess of unreacted linker to obtain a purer final product.
The BCA assay was performed using 6 blanks (3 blanks with milliQ water and 3 blanks with PBS 1X buffer), 2 BSA calibration lines (from 0.1 mg/ml to 1 mg/ml, with milliQ water) and 3 IgM conjugated samples (in PBS 1X).

2.5 DAR evaluation: RP-HPLC measure

In order to evaluate the number of drug molecules conjugated to every monomer of the IgM (DAR), I cannot perform a simple UV measure at the spectrophotometer because I don't have the exact coefficients needed. I opted for using a RP-HPLC measure, with a BioBasic™-4 column (diameter 1 mm, length 50 mm, pore size 300 Å, particle size 5 μm).
RP- HPLC is a chromatographic technique that allows the separation of various components from a liquid mixture. It's a qualification and quantification technique. Thanks to the solvent flow through the column, the sample can pass through this packed column and, according to their interaction with it, they are eluted at different times.

It's characterized by 2 phases: the stationary phase (HPLC column) and the mobile phase (solvent). The stationary phase generally is composed by a hydrophobic layer that allows the partial adsorption of the protein to the hydrophobic surface.

The column used for this analysis is composed by fused silica (matrix) derivatized with an aliphatic chain (butyl). This particular type of column is endcapped with trimethyl chlorosilane in order to uniform the hydrophobic layer. These aliphatic chains provide a hydrophobic surface on which the hydrophobic domains of the protein can distribute. The rest of the protein is in contact with the mobile phase.

The desorption of the proteins attached happens when a specific concentration of the organic solvent is reached. After the desorption, the protein elutes from the column. The most used organic solvents are acetonitrile and isopropanol.

In a mixture of different samples, higher the hydrophobicity of the sample, stronger the interaction with the column and so longer will be the elution.

The mobile phase is composed by a mixture of 2 solvents. Solvents A is made of 5 % of ACN and 0.1 % of TFA in mQ water. Solvent B is made of 75 % of ACN, 20 % of IPA and 0,09 % of TFA in mQ water. These 2 solvents are characterized by an increase in the organic content: from solvent A to solvent B the amount of ACN and IPA used is higher.

Generally, the loading of the sample is made with a mobile phase composed by a high percentage of the aqueous solvent and a low percentage of organic solvent in order to guarantee the absorption of every protein, including the most hydrophilic ones. After the absorption, the gradient of the mobile phase is modified by increasing the percentage of the organic solvent (ACN) to promote the elution of the protein, according to their hydrophilic/hydrophobic balance.

For this reason, the acetonitrile can be considered as the modifier of the mobile phase because it's able to modify the hydrophobicity of the mobile phase.

For this type of analysis, the column must be kept at 50°C during the entire analysis. In order to achieve a good separation of the analytes, the method is performed using a gradient of the mobile phase, starting with a higher content of the polar portion of the mobile phase and gradually increasing the apolar portion by time.

I calculated the DAR of a Lysine conjugate. The DAR is calculated as a ratio between the molar concentration of the drug and the molar concentration of the antibody.

I used 3 different samples: 1- pure Linker, 2- "Copy" conjugation and 3- IgM conjugated and completely reduced.

In particular:

1- It's the linker used for the conjugation. Its concentration is 4.6 mg/ml but I have to inject it at 0.1 mg/ml.

Linker Conc. (mg/mL)	4.6
Linker Conc. Injected (mg/mL)	0.1
ng injected	500
µL injected	5

2- The "Copy" conjugation is a conjugation set at the same condition of the real one (0.5 mg of IgM at 2.5 mg/ml). For this conjugation, I don't use any antibody but only Linker (5.65 µL) + DMSO (14.35 µL) + HEPES buffer pH 8 (180 µL). The reaction is conducted at 25 °C for 30 minutes. After this, the reaction must be brought to 1 mg/ml (which is the concentration of the reduction reaction). This reaction allows me to obtain the Linker hydrolysed (W/O NHS) in the same condition of the IgM, so the total amount of free drug.

Conc. conjugation reaction (mg/mL)	2.5
Linker Conc. in reaction (mg/mL)	0.12995
Linker Conc. Injected (mg/mL)	0.052
ng Linker injected	260
µL injected	5

3- After the real conjugation, an aliquot of the reaction is taken out and fully reduced with DTT 50 mM in TRIS HCl 50 mM buffer at pH 8. The reduction of the ADC is set at 1 mg/ml.

DTT is used because it can guarantee the complete reduction of the ADC. By the total reduction of the ADC, I can separate the free antibody to the Linker – Drug not reacted.

Conc. Conjugation reaction (mg/mL)	2.5
Conc. Reduction reaction (mg/mL)	1
Linker Conc. in reaction (mg/mL)	0.07903
ng injected	395
µL injected	5

After the HPLC runs, I need to integrate the peaks and derive the AUC and the retention time for each peak, with particular attention for:

- peak corresponding to the Linker – Drug not reacted (sample 3)

- peak corresponding to free Drug (Linker W/O NHS) (sample 2)

$$\% \textbf{ of free Linker} = \frac{(AUC\ Linker - Drug\ not\ reacted)}{(AUC\ Linker\ W/O\ NHS)} * 100$$

$$\% \textbf{ Linker conjugated} = [100 - (\%\ of\ free\ Linker)] * 100$$

Knowing the total amount of linker that I put in the conjugation reaction and the % of linker that reacted (and so that was conjugated), I can obtain the amount and therefore the mmoles of linker that have actually reacted. The DAR is calculated as reported below.

$$DAR = \frac{(Linker\ concentration)}{(IgM\ concentration)} = \frac{(mmoles\ of\ Linker\ reacted)}{(mmoles\ of\ IgM\ in\ reaction)}$$

2.6 In vitro assay - Viability assay

This assay allows to determine the concentration of ATP present in a cell culture (in vitro assay). ATP is a direct indicator of metabolically active cells. It can also be used for cytotoxicity assays, in order to determine the cytotoxic effect on specific cells. A CellTiter-Glo®[26] Luminescent kit is used. This kit is composed by CellTiter-Glo® Substrate and CellTiter-Glo® Buffer. The CellTiter-Glo® reagent is obtained by adding CellTiter-Glo® Buffer into CellTiter-Glo®. The reagent is then added directly to cells culture (ratio volume reagent : volume culture, 1:1), treated with a specific medium, and mixed for 2 minutes on an orbital shaker to induce cell lysis. The result is the generation of a luminescent signal that is directly proportional to the amount of ATP produced in the reaction. The reaction that occurs is the luciferase reaction. In presence of ATP and oxygen, the substrate luciferin is converted by the luciferase enzyme (that uses Mg^{2+} as co-factor) in oxyluciferin, with the consequent emission of light (blue or green light).
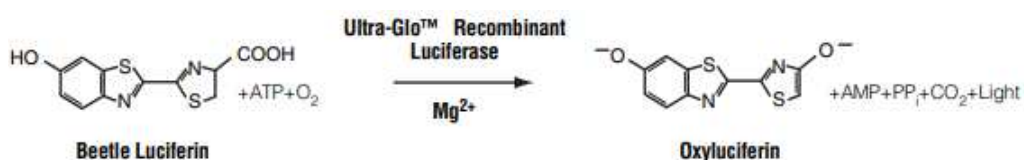

Figure 2.10: Bioluminescence assay. Luciferase reaction.

In a cell culture, higher is the number of viable cells, higher is the amount of ATP produced and so the amount of light emitted.

This assay is usually performed in a 96 Well Microplates and then the plate is read at the luminometer at 562 nm (wavelength of emission of luciferin).

2.6.1 – Cell lines tested

The conjugates were tested using two different cell lines: BXPC3 and Jurkat. BXPC3 is a human pancreatic cancer cell line. It was first isolated from the pancreas tissue of a 61-year-old female patient with adenocarcinoma in 1986. It grows for adhesion to the cell culture. This particular type of cell lack a KRAS mutation, that indicates pancreatic cancer. It's also associated with a high expression of angiogenic factor, such as IL-8 and PGE2. For my thesis purpose, it has been designed to express GPC-1 receptors (GPC1+).

Jurkat is an immortalized line of Human T lymphocyte. The cells can produce IL-2, that is responsible for immunity system cells replication. It was isolated from the peripheral blood of a 14-year-old boy affected with T cell Leukaemia. It grows for suspension in the cell culture. It doesn't express the GPC-1 receptors (GPC1-) and for this reason it's used as reference/negative control.

For my thesis project, 2 lysine conjugates and 1 cysteine conjugate were tested. 2 rounds of tests were done, at increasing concentration. For the 1° round, the concentrations tested for both conjugates on both cell lines were 100 nM; 10 nM; 1 nM; 0.1 nM and 0.01 nM. For the 2° round, the concentrations tested for the cysteine conjugate only on GPC1 + were 500 nM; 50 nM; 5 nM; 0.5 nM and 0.05 nM and for the lysine conjugates only on GPC1 + were 200 nM; 20 nM; 2 nM; 0.2 nM and 0.02 nM.

The 2° round was exploited at higher concentration in order to see if the conjugates were active also on the positive line (which was the desired effect).

RESULTS

3.1 IgM characterization

Before starting with the synthesis of the ADCs, it's important to characterize the different aliquot of IgM that were delivered.

The first thing to do is an UV measure at the spectrophotometer. This allows to determine the exact amount of the rate.

The wavelengths tested were: 280 nm, 350 nm, (280 - 350) nm and 248 nm. 280 nm is used for proteins, 350 nm for aggregates and impurities, 248 nm for the conjugates (for the drugs). It's important to evaluate the value at (280 – 350) nm in order to eliminate the contribution of the aggregates.

Every UV measure was done in triplicate. As a reference, I reported the average of every measure.

For the preparation of the samples:

- Blank: 300 µL of milliQ water

- Samples: 300 µL of IgM rate

Aliquot	280 nm	350 nm	(280 – 350) nm	248 nm
A	0.768	0.007	0.760	0.350
B	0.791	0.019	0.772	0.390
C	0.801	0.018	0.783	0.402

Table 3.1: Evaluation of the UV measure of the initial rate of IgM. λ tested: 280 nm, 350 nm, (280-350) nm, 248 nm. The values are the average of the measure performed in triplicate.

From the table 3.1, it's clear that there is a concordance among the values.

The values at (280 - 350) nm instead are used for the determination of the mg of IgM.

Applying the Lambert Beer Law, from the absorbance and the Specific extinction coefficient (1.32 mL*mg^{-1} *cm^{-1}), the concentration of the IgM can be obtained.

The optical path length is unitary.

With the concentration and the volume measured of the single aliquot, I can find the exact mg of IgM that can be used for the reactions.

Aliquot	Conc. , mg/mL	Volume, mL	Mg
A	0.570	1.831	1.044
B	0.585	1.850	1.082
C	0.593	1.910	1.133

Table 3.2: Determination of the mg of the rate of the IgM.

After the UV measure, an SDS-PAGE and a SEC analysis can be done.

These 2 techniques are optional because they involve the loss of mg of protein.

I did these two techniques only for the first rate, just as a reference.

For the SDS-PAGE, I used IgM pure, IgM reduced with 50 mM DTT in TRIS HCl buffer 50 mM, IgG reduced with 50 mM DTT in TRIS HCl buffer 50 mM (the IgG used was Trastuzumab, commercial Herceptin).
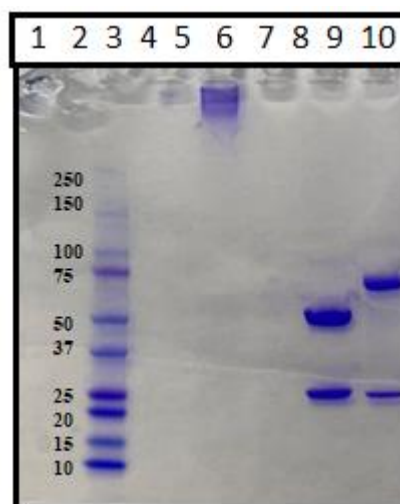

Figure 3.1: Lane 1: Empty; Lane 2: Empty; Lane 3: Dual Color Precision Plus Protein Standards Bio-Rad (250 kDa-10 kDa); Lane 4: Empty; Lane 5: Empty; Lane 6: IgM pure; Lane 7: Empty; Lane 8: Empty; Lane 9: IgG 50 mM DTT; Lane 10: IgM 50 mM DTT. Every sample was treated in non-reducing condition.

From the gel above (Figure 3.1), I can see that the IgM pure, due to its MW, doesn't run through the gel but remains up. It's also significant to consider the pattern of run of the IgM pure: it doesn't present any residue through the gel, meaning that it doesn't have fragments and important impurities.

The IgM and IgG reduced were used just as a reference, to compare the patterns of run. The IgG reduced (lane 9) and the IgM reduced (lane 10) have both two bands, the heavy chain and the light chain. The light chains are both located at 25 kDa instead the heavy chains are located one at 50 kDa (heavy chain of the IgG) and the other at 75 kDa (heavy chains of the IgM).

The chromatographic technique was done in order to evaluate the elution of the IgM. The column used was a Superose 12 10/300, for SEC. Technically it cannot separate an IgM, for its MW and hydrodynamic volume. The volume injected was 500 µL, corresponding to 0,2 mg of IgM. Also for this technique, an IgG was used as a reference.
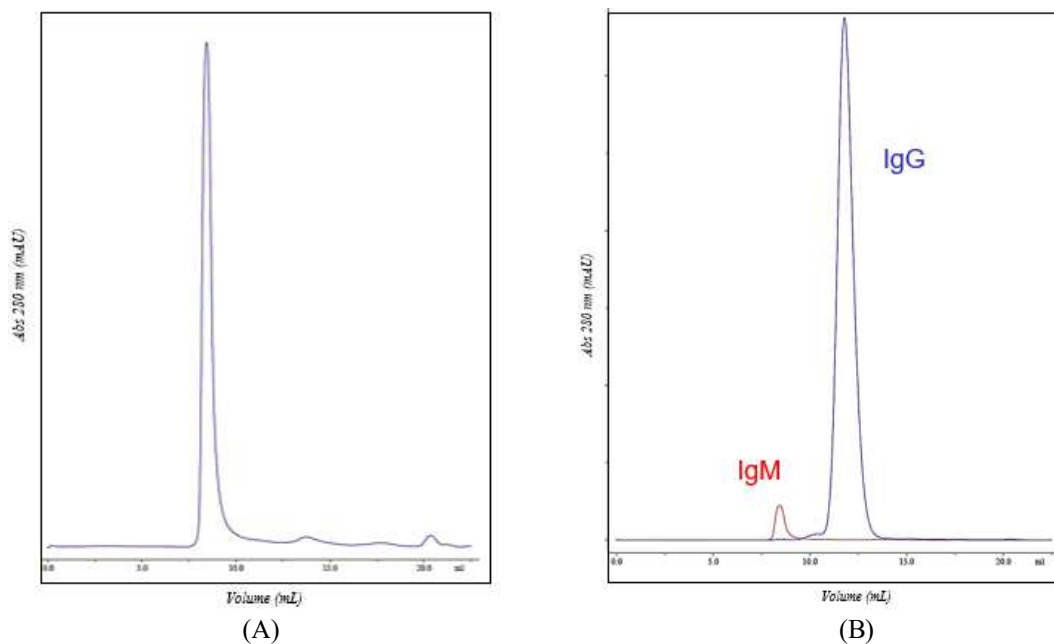

(A)   (B)

Figure 3.2: A. Elution chromatogram of the IgM pure obtained with SEC-FPLC. Elution peak (peak 1): 115 mAU (~ 8 mL). B. Elution chromatogram of the IgM pure (red peak) and the IgG pure (blue peak). The elution peak for IgM: 115 mAU (~ 8 mL). Elution peak IgG: over 1500 mAU (~ 12 mL).

For both chromatograms, the elution volume and the absorbance at 280 nm (measured in mAU) were evaluated. The elution volume can be compared to the retention time.

In this case, considering Figure 3.2 (A)., the IgM chromatogram shows 2 main peaks:

- peak 1: 8 mL

- peak 2: 14 mL

Only peak 1 has an observable absorbance: 115 mAU. This absorbance is very low due to small amount of protein injected.

Considering Figure 3.2 (B), the peak corresponding to the IgM elutes before the peak corresponding to the IgG. The principle behind this technique is that bigger molecules elute before the smaller ones because of the hydrodynamic volume and the penetration into the resin's pores. In this case the MW of an IgM is higher than the MW of an IgG and so its dimension: the IgM is bigger than the pores of the column, so it's not retained from it, and it elutes with the dead volume.

In addition to that, it's useful to do a BCA assay on the initial aliquot in order to verify its concentration. The sample was tested after 1:3 dilution with milliQ water.

	IgM
Concentration at the spectrophotometer	0.7126 mg/mL
Concentration with the BCA assay	0.8435 mg/mL

Table 3.3: Comparison between different techniques for the determination of the concentration of the initial aliquot of the IgM.

The wavelengths analysed were: (280 – 350) nm for the spectrophotometer measure and 562 nm for the BCA assay.

It's clear that there is a discrepancy between these results: the most accurate one is the one obtained with the BCA assay.

Thanks to these 4 techniques, I can understand the main characteristic of the IgM, including the amount and the purity.

3.2 SDS-PAGE of the optimization of the disulphide bonds reduction

The sample were tested in non-reducing condition because they were all after the reduction step. The results of the reduction with different concentration of TCEP shows that the optimal concentration of TCEP is 1 mM, even though the reduction wasn't fully complete. With 3 mM and 10 mM of TCEP, the IgM tends to fragmentate. The reduction with 50 mM of DTT is used only when the total reduction of the IgM is required. With this concentration of the reducing agent, the reduction is not only of the interchain disulphide bonds but also of the intrachain disulphide bonds. In order to say that the reduction is complete, I need to visualize the following bands on the gel:

- J chain (18 kDa): it's generally present in a pentameric IgM
- Light chain (approximately 25 kDa)
- Heavy chain (approximately 75 kDa)

For the disulphide bonds reduction, I expected to see only the light and the heavy chain, not the J chain, because I wanted a partial reduction (only of the interchain) and not a fully reduction of the antibody.
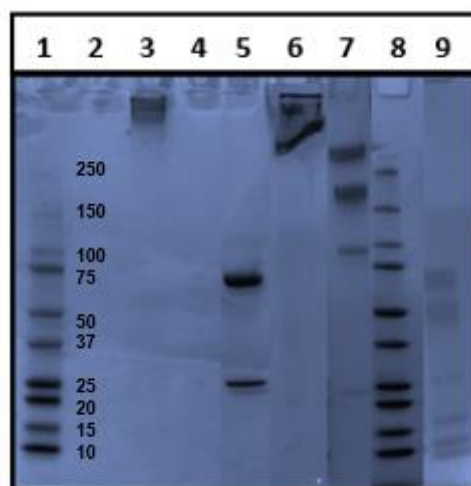
Figure 3.3: Lane 1: Dual Color Precision Plus Protein Standards Bio-Rad (250 kDa-10 kDa); Lane 2: Empty; Lane 3: IgM pure (not reduced); Lane 4: Empty; Lane 5: IgM DTT 50 mM; Lane 6: IgM 7.814 μM; Lane 7: IgM TCEP 1 mM; Lane 8: IgM TCEP 3 mM; Lane 9: IgM TCEP 10 mM. Every sample was treated in non-reducing condition.

As we can see from the gel above (Figure 3.3), the complete reducing is obtained with DTT 50 mM (only 2 bands can be visualized). At increasing concentration of TCEP, a fragmentation of the IgM is seen. With TCEP 7.814 μM, IgM is still entire because the reduction pattern is the same of the IgM pure (Lane 3). With TCEP 1 mM we can notice that there isn't any fragmentation. This can be seen comparing the reduction pattern of IgM TCEP 1 mM (Lane 7) with IgM TCEP 3 mM and 10 mM (Lane 8 and Lane 9).

More specifically, by looking at the reduction pattern with TCEP 1 mM (lane 7), there are 4 bands visible:

- 25 KDa -> L chain

- 75 KDa -> H chain

- 150 KDa -> HH chains

- 250 KDa -> HHLL chains

This means that the reduction of the interchain disulphide bonds with TCEP 1 mM is not complete because I can also see HH chains and HHLL chains, that are visible if the interchain disulphide bonds are intact. In fact, HHLL chains correspond to the entire monomer.

3.3 SEC of the optimization of the disulphide bonds reduction

After the reduction step, the reaction is purified from the excess of the reducing agent, through SEC-FPLC. It's fundamental to remove the not reacted reducing agent because it can induce new reduction in the antibody. The column used is the Superose 12 10/300, equilibrated in PBS pH 7.4 + 2 mM EDTA.

The following chromatogram is a comparative chromatogram, obtained overlapping the single chromatograms of the purification of the reduction reaction, exploited with TCEP 1 mM, 3mM and 10 mM.
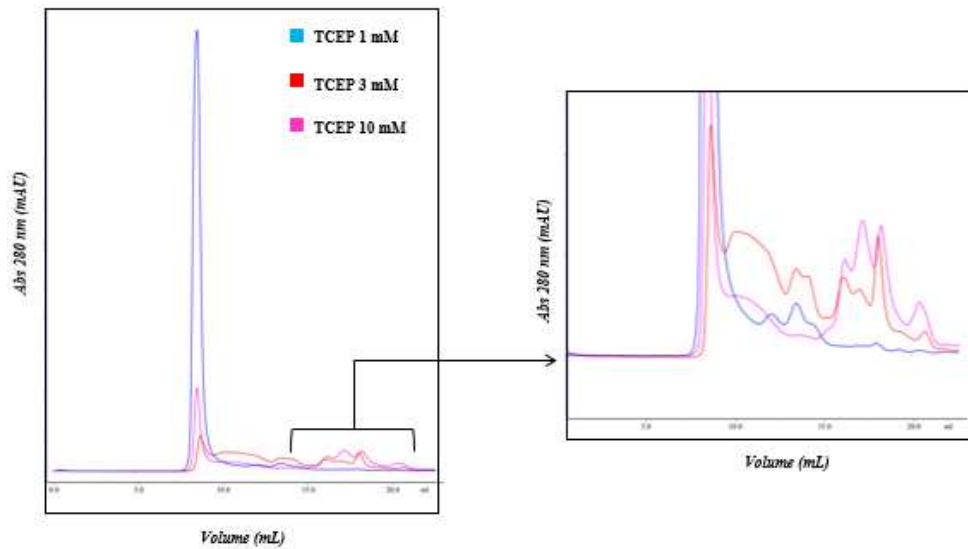

Figure 3.4: Comparative chromatogram of the reduction with TCEP 1 mM, 3 mM and 10 mM. On the right, there is a zoom of the chromatogram that underline the aggregation. Obtained with SEC-FPLC.

The aim is to underline and confirm the differences in the reduction behaviour of TCEP, observed also in the SDS-PAGE (§ 3.2). The zoom of the chromatogram represents the region of the secondary peaks, where smaller fragments are eluted. By looking at it, it's possible to say that the blue chromatogram (TCEP 1 mM) is the best one because the pattern is more linear, compared to TCEP 3 mM and TCEP 10 mM.

It's also important to observe the ratio main peak/secondary peaks: the chromatogram of TCEP 1 mM has the highest ratio, meaning that the amount of fragments (or excess of reducing agent) is very low, confronted with the amount of IgM eluted. The chromatograms of TCEP 3 mM and TCEP 10 mM show that the ratio main peak/secondary peaks are very low.

3.4 SDS-PAGE of the reduction comparison between IgG and IgM

From the SDS-PAGE of the disulphide bond reduction (Figure n. 3.3), it's unclear the reason of the fragmentation of the IgM. For this reason, another SDS-PAGE analysis was executed. In this case I wanted to test the reduction of the IgG with the same equivalents of the reducing agent used for the reduction of the IgM, to search for a common pattern of reduction. The results were obtained considering the same equivalents of reducing agent TCEP between IgM and IgG to evaluate its behaviour on 2 different proteins. The mmolar concentration was calculated afterwards.

	Equivalents	mmolar concentration
IgM	1152 Eq	TCEP 3 mM
IgM	3839 Eq	TCEP 10 mM
IgG	1152 Eq	TCEP 20 mM
IgG	3839 Eq	TCEP 66.6 mM

Table 3.4: Correlation between equivalents and mmolar concentration of IgM and IgG.

From the gel (Figure 3.5) it's clear that the problem is not the reducing agent used because with the equivalents used the IgG gives a clear and comparable reduction pattern for both the equivalents used. In this case we can see multiple bands corresponding not only to the Light Chain and the Heavy Chain because the equivalents used were more than the equivalents used for the classical interchain disulphide bonds reduction for the IgG.

The same thing happens for the IgM: the reduction pattern obtained with the 2 amounts of equivalents of TCEP used is the same. It's obvious that by increasing

the amount of TCEP used in the reduction reaction it's possible to obtain a progressively more complete reduction. But the IgM should not fragment.

I can conclude that the reduction differences notated between using TCEP 1mM, 3 mM and 10 mM are related to intrinsic characteristics of the protein and not to the type and equivalents of reducing agent used.
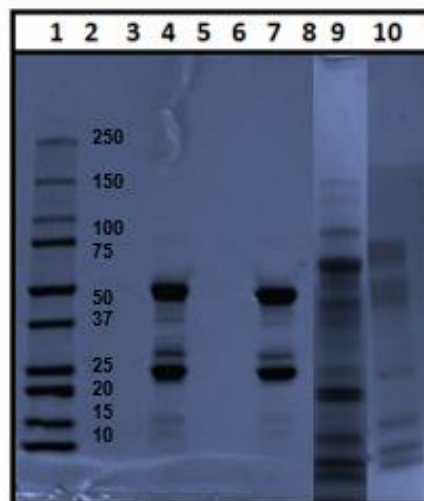

Figure 3.5: Lane 1: Dual Color Precision Plus Protein Standards Bio-Rad (250 kDa-10 kDa); Lane 2: Empty; Lane 3: Empty; Lane 4: IgG 1152 Eq (TCEP 20 mM); Lane 5: Empty; Lane 6: Empty; Lane 7: IgG 3839 Eq (TCEP 66.6 mM); Lane 8: Empty; Lane 9: IgM 1152 Eq (TCEP 1 mM); Lane 10: IgM 3839 Eq (TCEP 3 mM).

Every sample was treated in non-reducing condition.

3.5 Cysteine conjugates analysis through SEC-FPLC and UV measure

The cysteine conjugates were produced following the Double Step Synthesis, described in §2.3.

	Conjugate	Eq TCEP/IgM	TCEP Conc.
REDUCTION	Cysteine 1	3	7.814 µM
	Cysteine 2	384	1 mM

Table 3.5: Cysteine conjugates characteristics – Reduction Step

	Conjugate	Eq Linker/IgM
CONJUGATION	Cysteine 1	6
	Cysteine 2	25 (Excess/SH: 1.25)

Table 3.6: Cysteine conjugates characteristics – Conjugation Step

After the first step of the reaction (reduction step), the conjugates were purified from the excess of unreacted reducing agent through SEC (SE-FPLC). The column used was a Superose 12 10/300, equilibrated with PBS pH 7.4 + 2 mM EDTA. Following I'm reporting the chromatograms of the purification, registered at 280 nm.
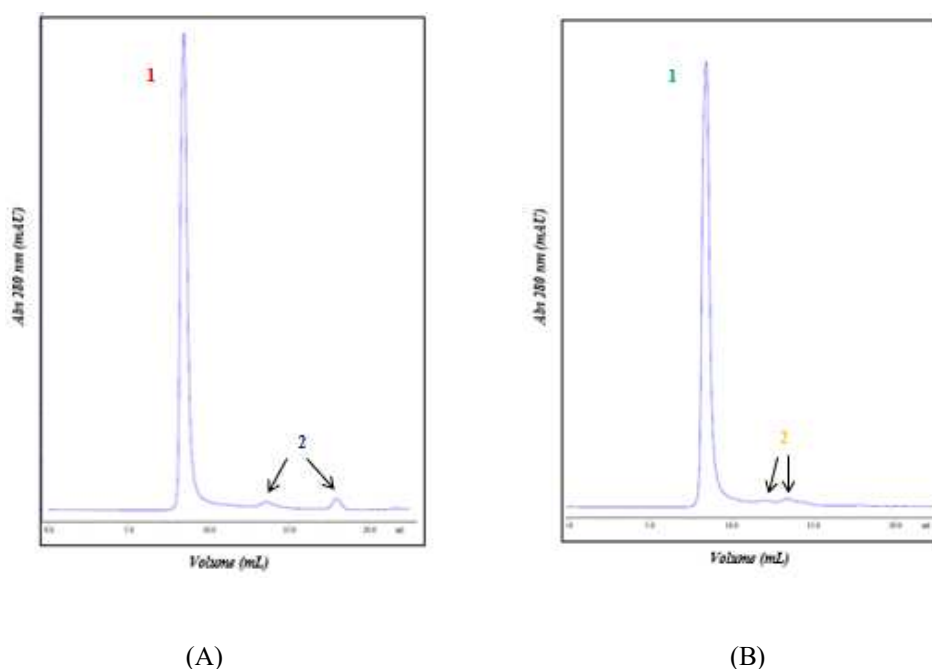

(A)   (B)

Figure 3.6: A- Chromatogram of the purification of the conjugate with TCEP 7.814 μM. Main peak in red (peak 1). Secondary peaks in blue (peaks 2). B- Chromatogram of the purification of the conjugate with TCEP 1 mM. Main peak in green (peak 1). Secondary peaks in yellow (peaks 2). Obtained with SEC-FPLC.

Considering the chromatogram A (Figure 3.6), the main peak (peak red) corresponds to the IgM reduced and purified. IgM elutes at ~ 8 mL, giving a milli-absorbance of 500 mAU. Secondary peaks (peaks blue) can be associated with the unreacted reducing agent or protein fragments. Since the MW of TCEP is lower

than the MW of the IgM, they elute afterwards (~ 14 mL and ~ 18 mL). It's important to underline the fact the IgM elutes as a unique peak, even after the reduction with TCEP, thanks to hydrophobic interactions (strong) between the IgM's chains.

Also for chromatogram B (Figure 3.6), the main peak (peak green) is the IgM after reduction and the secondary peaks (peak yellow) are residues of the reducing agent. The elution time for peak green is the same of peak red (~ 8 mL) instead the elution time for peaks yellow are slightly different (~ 13 mL and ~ 14 mL).

A purification was executed also after the Second Step (conjugation step). In this case, it's important in order to eliminate the excess of (Linker + Drug) not conjugated and to exchange the reaction buffer.

The column used is always a Superose 12 10/300, for SEC (SEC-FPLC). The solvent used for the equilibration is PBS 1X buffer at pH 6.5.
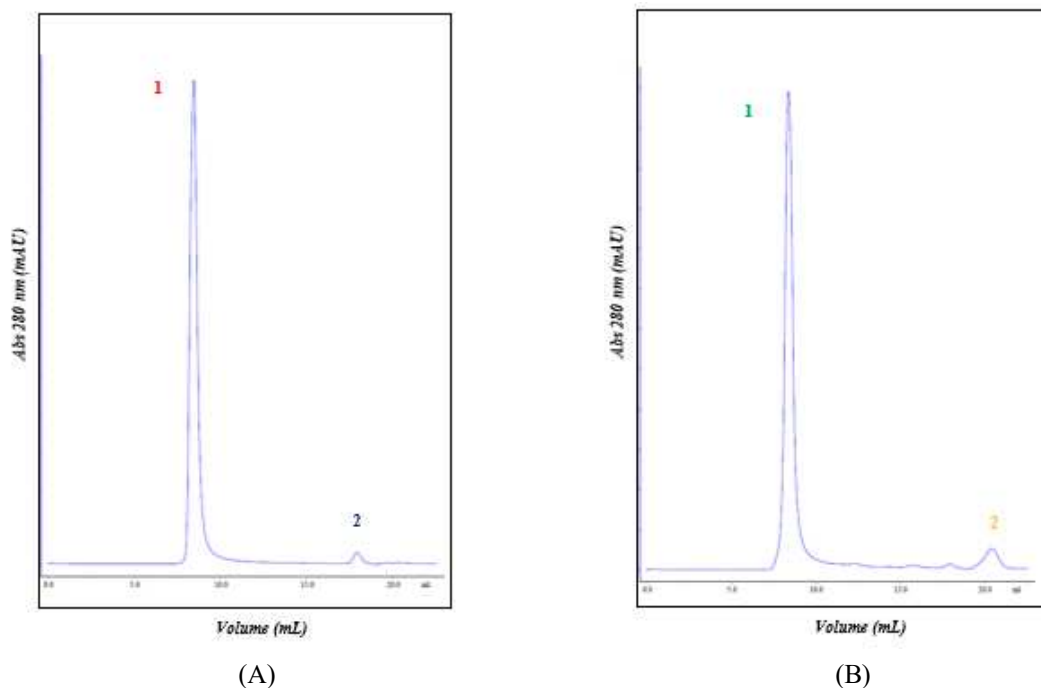

(A)   (B)

Figure 3.7: A- Chromatogram of the purification of the conjugate with 6 Eq of Linker. Main peak in red (peak 1). Secondary peak in blue (peaks 2). B- Chromatogram of the purification of the conjugate with 25 Eq of Linker. Main peak in green (peak 1). Secondary peak in yellow (peaks 2). Obtained with SEC-FPLC.

Also for these chromatograms, the main peak (peak 1 for both) is the conjugate purified. The milli-absorbance registered is 350 mAU for the chromatogram A and 320 mAU for chromatogram B. The elution volume instead is the same for both is around 8 mL.

Even though the amount of linker used was higher for the second conjugate (6 Eq vs. 25 Eq), the elution volume of peak 1 is the same. This can be confirmed by the peaks 2: the peak 2 of the chromatogram B is higher than the peak 2 of the chromatogram A, meaning that a higher amount of linker was eluted and so not conjugated. This is visible also in the comparative chromatogram in Figure 3.8.
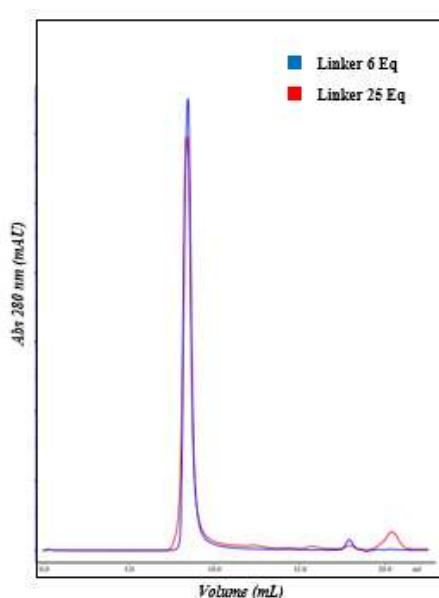

Figure 3.8: Comparative chromatogram of the 2 conjugates after purification in SEC-FPLC. Red chromatogram: Linker 6 Eq. Blue Chromatogram: Linker 25 Eq.

In order to see if any conjugation occurred, a UV measure at the spectrophotometer can be useful. The wavelengths tested were: 280 nm (for proteins), 350 nm (for aggregates) and 248 nm (for drug). It's important to compare the values at 248 nm before and after conjugation: if this value increases, it means that there is a higher contribute of the drug, showing conjugation.

Another value that can be considered is the ratio between the absorbance at 280 nm and the absorbance at 248 nm: if this ratio decreases and moves towards unity, it means that the conjugation occurred. The values in table 3.7 are the average.

For the preparation of the samples:

- Blank: 300 µL of PBS pH 7.4 + EDTA 2 mM (before conj.) and 300 µL of
    PBS 1X buffer at pH 6.5 (after conj.)

- Samples: 300 µL of reaction purified

	Abs before conjugation	Abs after conjugation
Cysteine 1		
280 nm	0.242	0.289
350 nm	0.018	0.002
(280 – 350) nm	0.236	0.287
248 nm	0.099	0.129
280 nm / 248 nm	2.44	2.24
Cysteine 2		
280 nm	0.478	0.242
350 nm	0.007	0.009
(280-350) nm	0.470	0.233
248 nm	0.202	0.220
280 nm / 248 nm	2.37	1.10

Table 3.7: Absorbance values for both Cysteine conjugates, before and after conjugation.

Looking at Table 3.7, for both conjugates, I notice an increase in the absorbance at 248 nm from before conjugation to after conjugation. The increase, calculated as the ratio between the absorbance at 248 nm after conjugation and before conjugation, is similar: for conjugate 1 (TCEP 7.814 µM) is equal to 1.30 and for conjugate 2 (TCEP 1 mM) is equal to 1.09. Considering the value (280 nm / 248 nm) it decreases from before conjugation to after conjugation: it means that the conjugation occurred.

## 3.6 Lysine conjugates analysis through SEC-FPLC and UV measure

The lysine conjugates were produced following the Single Step Synthesis, described in §2.4. After the conjugation, the ADCs were purified using a Superose 12 10/300, for SEC (SEC-FPLC), equilibrated in PBS 1X buffer pH 6.5. The purification is exploited to eliminate the excess of (Linker + Drug) not loaded and to exchange the reaction buffer.

Conjugate	Eq Linker-Drug / Eq IgM
Lysine 1	40 (Excess/ Eq IgM: 2)
Lysine 2	60 (Excess/ Eq IgM: 3)
Lysine 3	70 (Excess/ Eq IgM: 3.5)

Table 3.8: Lysine conjugates characteristics – Conjugation Step

(A)   (B)
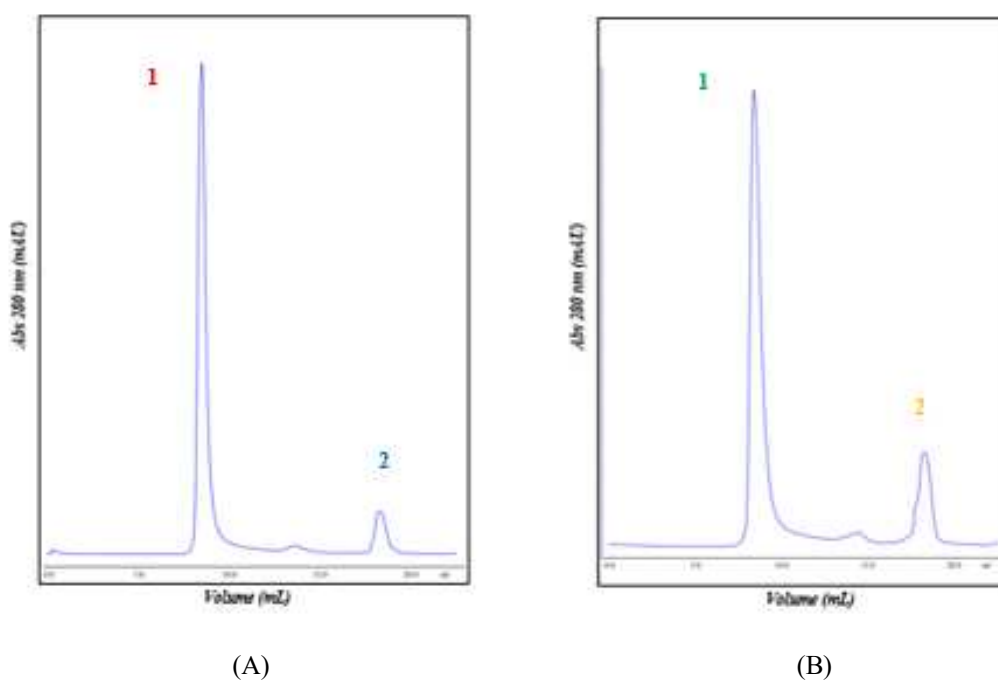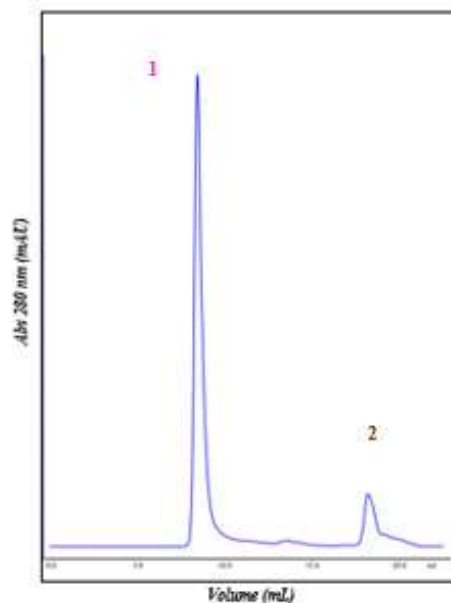
(C)

Figure 3.9: A- Chromatogram of the purification of the conjugate with 40 Eq of linker. Main peak in red (peak 1). Secondary peak in blue (peak 2). B- Chromatogram of the purification of the conjugate with 60 Eq of linker. Main peak in green (peak 1). Secondary peak in yellow (peak 2). C- Chromatogram of the purification of the conjugate with 70 Eq of linker. Main peak in pink (peak 1). Secondary peak in brown (peak 2). Purified in SEC-FPLC.

As for cysteine conjugates, also for lysine conjugates the main peaks (peaks 1) and the secondary peaks (peak 2) elute at around the same elution volume (~ 8 mL for peaks 1 and ~ 18 mL for peaks 2).

3.7 DAR evaluation with RP-HPLC measure

The DAR was evaluated only for Lysine conjugate 1 (40 Eq of Linker - Drug). For the run parameters and the instrument setting, look at paragraph §2.5.
The integration of the peaks obtained in RP-HPLC allowed to determine the DAR of this conjugate.

After the integration of the peaks, the parameters that must be observed are:

- retention time

- AUC

In the following table I'm reporting the values obtained.

	ng loaded	Rt (min)	AUC (mAU x min)
IgM conjugated and reduced	395 ng	6.035 min	225.3
Linker w/o NHS	260 ng	6.146 min	371.1

Table 3.10: Summary data of the RP-HPLC analysis.

The IgM conjugated and reduced corresponds to the Linker – Drug not reacted. Instead the Linker W/O NHS corresponds to the free drug.

In particular, I loaded 5 μL of both: the IgM conjugated and reduced reaction was injected at 0.07903 mg/ml (equals to 395 ng loaded) instead the Linker W/O NHS reaction was injected at 0.052 mg/ml (equals to 260 ng loaded).
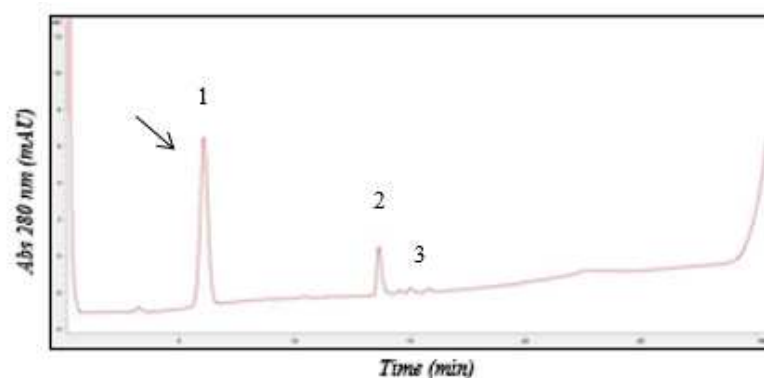

Figure 3.10: Chromatogram of the hydrolysed linker (W/O NHS). Reaction conducted in the same condition of the conjugation reaction ([ ] = 2.5 mg/mL, in HEPES buffer 50 mM at pH 8 and 10 % of DMSO). Peak signed with an arrow: linker W/O NHS. Retention time: 6.146 min. AUC: 371.1 (mAU x min).
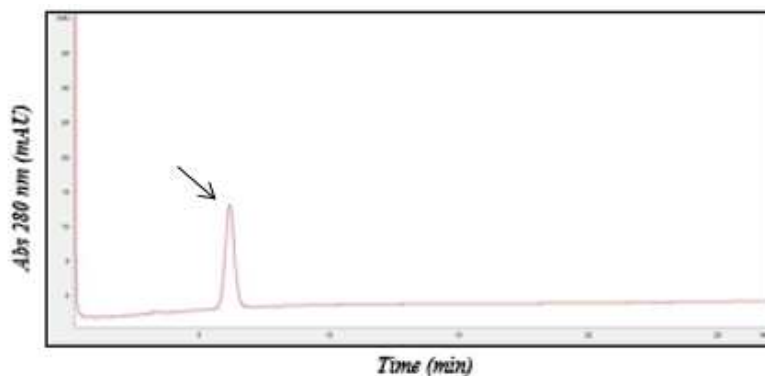
Figure 3.11: Chromatogram of the IgM conjugated and reduced. Conjugation reaction set up at 2.5 mg/mL, in HEPES buffer 50 mM at pH 8 + 10 % DMSO. Reduction reaction set up at 1 mg/mL, with 50 mM DTT, in TRIS HCl 50 mM buffer. Peak signed with an arrow (peak 1): IgM conjugated and reduced. Retention time: 6.035 min. AUC: 225.3 (mAU x min).

From figure 3.11, the chromatogram shows more than one peak. In order to understand which one is the interested peak, I have to consider the retention time of the peaks and confront them with the retention time of the peak of the chromatogram of the Linker W/O NHS (Figure 3.10). Peak 1 is the linker that had reacted in the conjugation reaction instead Peak 2 and 3 are the IgM reduced (peak 2 is the heavy chain instead peak 3 is the light chain).

In this case, peak 1 is the peak that has the retention time similar to the peak of the chromatogram of the Linker W/O NHS.

In order to calculate the % of free linker, I have to apply the formula reported in paragraph §2.5.

The aim was a DAR of 20, considering 4 molecules of cytotoxic drug per monomer.

The DAR obtained was 15.5 (3.1 molecules of cytotoxic drug per monomer).

Considering that the amino group that reacts and can be conjugated are the most exposed at the solvent, this value is acceptable.

3.8 BCA for the determination of the concentration of the conjugates

The BCA assay was performed in order to obtain the total yield of the reactions.

Cysteine conjugate with TCEP 7.814 µM		
mg pre reduction	**mg post reduction**	**Total yield**
0.5	0.354	70.8 %
mg pre conjugation	**mg post conjugation**	**Total yield**
0.357	0.231	64.7 %

Table 3.11: BCA assay results for Cysteine conjugate using TCEP 7.814 µM.

Cysteine conjugate with TCEP 1 mM		
mg pre reduction	**mg post reduction**	**Total yield**
0.7	0.341	48.7 %
mg pre conjugation	**mg post conjugation**	**Total yield**
0.353	0.205	58.1 %

Table 3.12: BCA assay results for Cysteine conjugate using TCEP 1 mM.

Lysine conjugate with Linker – Drug 102.8 µM (40 Eq of Linker - Drug)		
mg pre conjugation	**mg post conjugation**	**Total yield**
0.526	0.232	44.1 %

Table 3.13: BCA assay results for Lysine conjugate with 40 Eq of Linker-Drug in reaction.

Lysine conjugate with Linker – Drug 156.7 µM (60 Eq of Linker – Drug)		
mg pre conjugation	**mg post conjugation**	**Total yield**
0.244	0.00895	3.7 %

Table 3.14: BCA assay results for Lysine conjugate with 60 Eq of Linker-Drug in reaction.

3.9 Conjugates analysis in vitro

The vitro test was conducted using the CellTiter-Glo®[26] Luminescent kit. The conjugates tested were the following:

- Lysine conjugate n.1 (Conc.= 0.7197 mg/ml; µg= 23.17)

- Lysine conjugate n.2 (Conc.= 0.1517 mg/ml; µg= 8.95)

- Cysteine conjugate n.2 (Conc.= 0.177 mg/ml; µg= 205.32)

All conjugates were tested on 2 different cell lines: one expressing the receptor for GPC1 (Rec+) and the other one not expressing the receptor (Rec -).
In the following table is reported the excepted activity vs. observed activity of the conjugates.

Conjugate type	Expected Activity		Observed Activity	
	Rec +	Rec -	Rec +	Rec -
Lysine 1	YES	NO	NO	NO
Lysine 2	YES	NO	NO	NO
Cysteine 2	NO	NO	YES	YES

Table 3.15: Summarizing table of the activity of the conjugates tested (expected activity vs. observed served activity). Rec +: Cell line expressing the receptor; Rec -: cell line not expressing the receptor.

As we can see from the table 3.15, I expected that the lysine conjugates were active only on the Rec + cells and not also on the Rec – cells. In this way, I could have demonstrated the active targeting function mediated from the lysine conjugates: if the activity was directed only on the Rec + cells, it would have meant that the conjugates were able to recognize specifically the receptor.
Instead, these conjugates showed no activity on both cell lines. The inactivity on the Rec – is a positive thing because it means that the targeting can be mediated from the recognition of the receptor, and it's not related to passive targeting.

The cysteine conjugate instead showed activity on both cell lines: it's difficult to explain if the activity is mediated from the interaction with the receptor or from the dimension of the conjugate. Big conjugates, such as ADCs, are attracted to the tumour site because of EPR effect. This effect can explain the activity on Rec- cells. Cysteine conjugates, after the reduction step, can lose the ability to activate the complement system (IgG doesn't preserve this function). This means that the activity showed on Rec- cells can be only for the free drug attached to the antibody.

The graphs showing the results are shown below. 2 rounds of cytotoxicity test were executed. For the first round of tests both cell lines (Rec+ and Rec-) were used. The concentration employed for both conjugate types were 100 nM; 10 nM; 1 nM; 0.1 nM; 0.01 nM. In this first round of test, they wanted to see the specific activity of these conjugates.
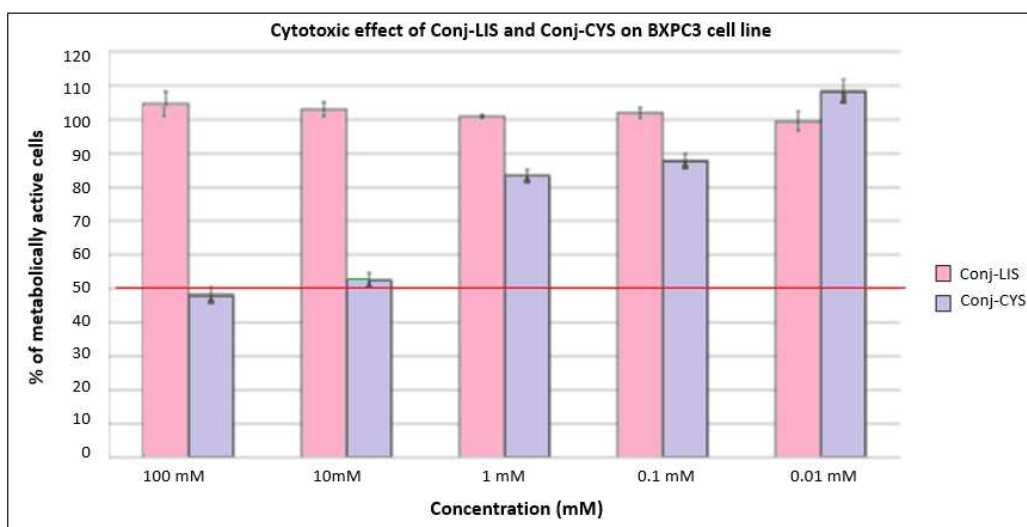

Figure 3.12: Histogram representative of the effect of both conjugates on BXPC3 cell line.
Pink: Lysine conjugate   Purple: Cysteine conjugate   Red line: IC_{50} value
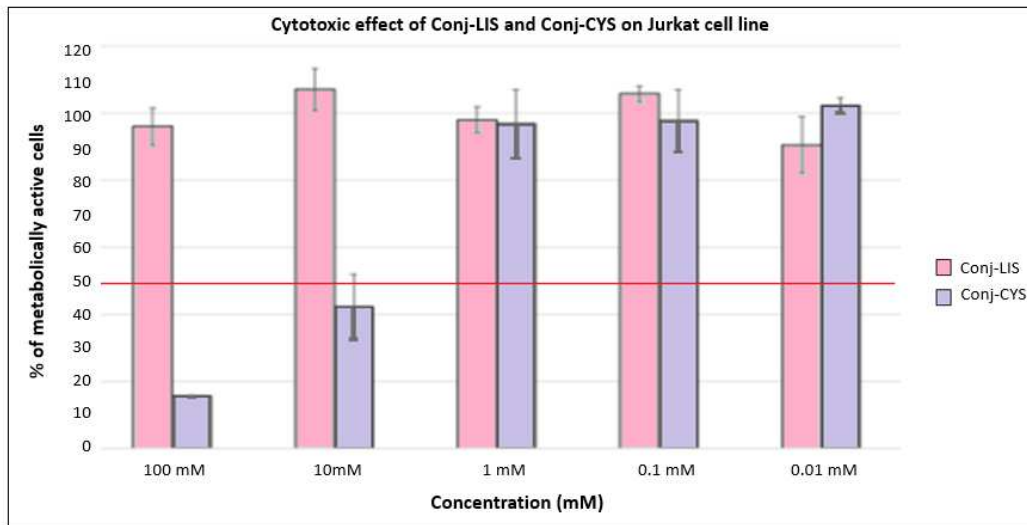
Figure 3.13: Histogram representative of the effect of both conjugates on Jurkat cell line.

Pink: Lysine conjugate   Purple: Cysteine conjugate   Red line: IC_{50} value

Figure 3.14: Histogram representing the cytotoxic effect of Lysine conjugates on BXPC3 Cell Line and Jurkat cell line.

Orange: BXPC3 cell line   Yellow: Jurkat cell line   Red line: IC_{50} value
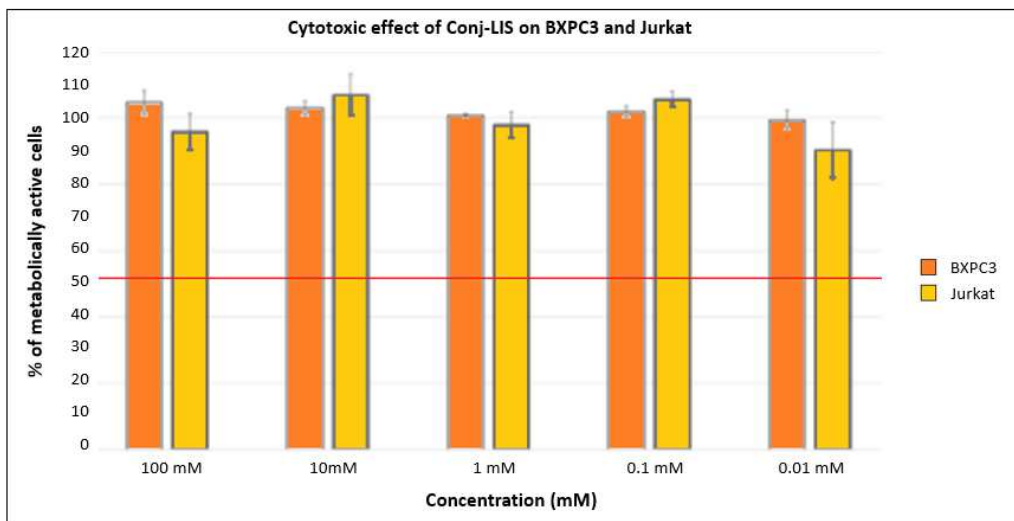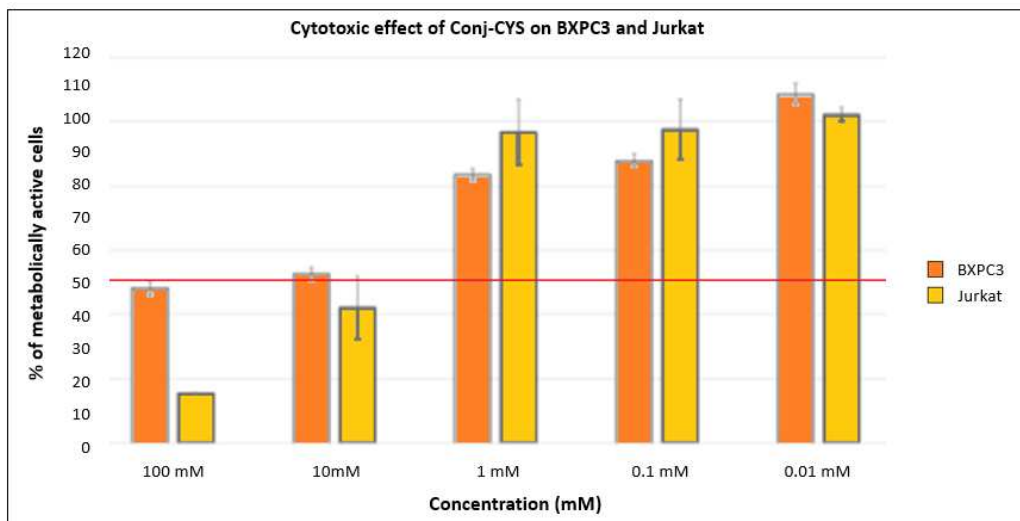
Figure 3.15: Histogram representing the cytotoxic effect of Cysteine conjugate on BXPC3
Cell Line and Jurkat cell line.

Orange: BXPC3 cell line   Yellow: Jurkat cell line Red line: IC_{50} value

These histograms were realised putting the concentration of the conjugates on the x-axis as the independent variable instead the % of metabolically active cells on the y-axis as the dependent variable.

The % of metabolically active cells indicates the rate of cells that remain active before and after the treatment. At the beginning the % of metabolically active cells is going to be 100, instead after 48 h it should decrease because of the effect mediated by the conjugates.

In particular, the IC_{50} was evaluated. It's the half maximal inhibitory concentration, the measure of potency of a substance in inhibiting a specific biological function. In this case, it's the concentration of ADCs at which the 50 % of the cells tested remain active after the 48h treatment. It can also be considered as a measure of the activity of the ADCs.

As we can see from the Figure 3.12, none of the conjugates at any concentration reaches the IC_{50} value. For all the concentrations tested, the % of metabolically active cell remains above 50 %. It means that none of the conjugates are active on

BXPC3 cell line (Rec +), in this specific range of concentration. From this histogram it's possible to see that the lysine conjugates maintain a % of metabolically active cells around 100 %, for every concentration tested. Instead for the cysteine conjugate it's possible to see a reduction of the % of metabolically active cells as the concentration of the ADC tested increases, but always above 50 % (not significant).

As regards the figure 3.13, the activity on Jurkat cell line (Rec-) was tested for both conjugate types. The behaviour of the Lysine conjugate is the same as on BXPC3 cell line. All the columns remain higher than the IC_{50} line. Even for the cysteine conjugate, the behaviour is similar as on BXPC3 cell line. In this case the reduction of the % of metabolically active cells is more pronounced, specifically between 10 nM and 100 nM. It was possible to determine the IC_{50} value (6.391 nM).

Figure 3.14 and Figure 3.15 are summarising the behaviour of each conjugate on both cell lines. In particular, considering figure 3.14, it's clear that the Lysine conjugates are inactive both on BXPC3 cell line and Jurkat cell line. Instead, considering figure 3.15 for the cysteine conjugate, for both cell lines, it's visible that the % of metabolically active cells decrease at increasing concentration. But the activity is positive only on the Jurkat cell line (Rec -).

For the second round of tests only Rec+ cell line was used. The concentration employed for lysine conjugates were 200 nM; 20 nM; 2 nM; 0.2 nM; 0.02 nM. The concentration employed for cysteine conjugates were 500 nM; 50 nM; 5 nM; 0.5 nM; 0.05 nM. This second round instead was used as a confirmation of the results obtained from the first round.
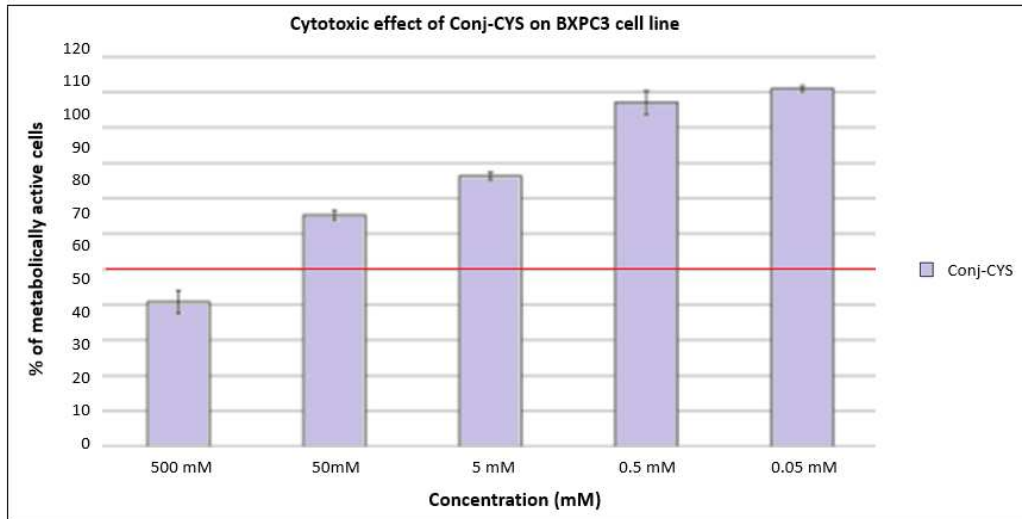
Figure 3.16: Histogram representative of the cytotoxic effect of Cysteine conjugate on
BXPC3 cell line.

Concentration tested: 500 nM; 50 nM; 5 nM; 0.5 nM; 0.05 nM.

Red line: IC_{50} value.
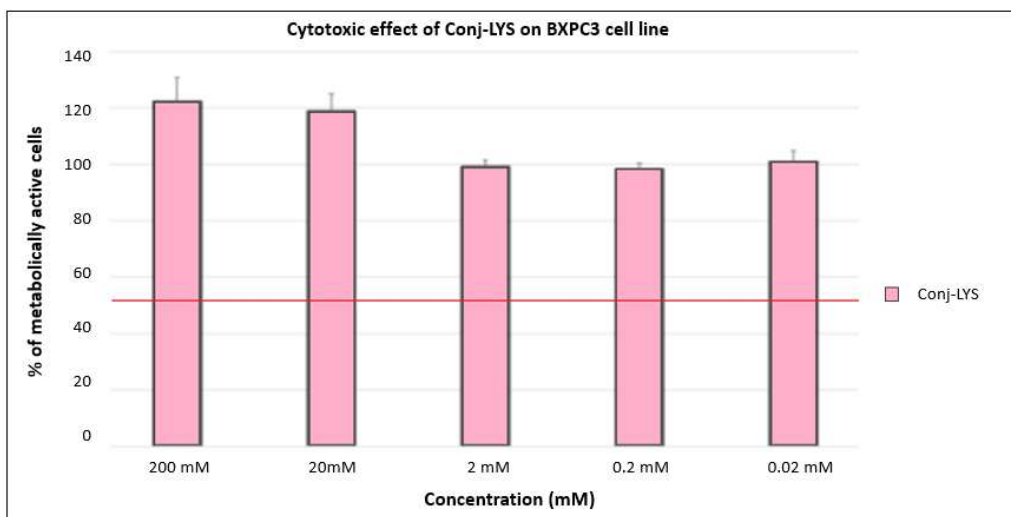
Figure 3.17: Histogram representative of the cytotoxic effect of Lysine conjugates on
BXPC3 cell line.

Concentration tested: 200 nM; 20 nM; 2 nM; 0.2 nM; 0.02 nM.

Red line: IC_{50} value

Comparing Figure 3.16 and Figure 3.17, it's possible to confirm that the Lysine conjugate isn't active on BXPC3 cell line because the IC_{50} value isn't reached instead the Cysteine conjugate shows activity on this cell line, with an IC_{50} value equals to 275.9 nM.

It's also important to underline the fact that the Cysteine conjugate induces more cytotoxicity on Jurkat cell line than on BXPC3 cell line (6.391 nM vs. 275.9 nM). In order to verify these hypothesis, in vivo tests should be done, testing both types of conjugates.

4.0 DISCUSSION

The advantages that come with the use of an antibody drug conjugate are multiple. The aim of my thesis project is to synthetize a new type of antibody drug conjugate, using an IgM as the antibody, instead of the classic IgG.

I applied two different synthesis techniques: double step synthesis (conjugation at the cysteine, through reduction of interchain disulphide bonds) and single step synthesis (conjugation at the lysine, through ε-amino group). Firstly, the IgM was characterized. This step was fundamental because it gave important information, such as concentration and purity of the protein.

For the IgM, it was used: UV measurement at the spectrophotometer, SDS-PAGE, SEC and BCA assay.

Through the UV measurements, performed at different wavelengths (280 nm, 248 nm, 350 nm), the initial concentration of the IgM was calculated. Knowing the exact concentration is fundamental in order to determine the mg of IgM available for the synthesis. These values can be seen in Table 3.1 and Table 3.2.

The SDS-PAGE was done in non-reducing condition by using the IgM in native and reduced conformation. By working in native condition, it was possible to confirm that the excessive dimension of the IgM couldn't allow the permeation through the gel. A clear band was visible at the top of the gel. When reduced, the IgM showed a pattern of bands similar to that of an IgG. It was possible to visualize the light chain (approximately at 25 kDa) and the heavy chain (approximately at 75 kDa).

Size Exclusion Chromatography was done in order to evaluate the elution and the chromatographic profile of the native IgM. Using an IgG as a reference, the IgM elutes at lower retention time with respect to the IgG due to its higher MW.

The BCA assay is important to determine the exact concentration of any sample. The results are reported in Table 3.3. The concentration obtained with the spectrophotometer and the concentration obtained with the BCA assay were different.

The concentration of the aliquot of IgM determined with the spectrophotometer was 0.7126 mg/mL.

Instead the concentration of the IgM evaluated with a BCA assay was 0.8435 mg/mL.

The first type of synthetized conjugates were obtained by coupling the drug to the cysteine residues obtained after the reduction of the interchain disulphides. For this type of conjugation, it's important to consider the reducing agent and the Linker-Drug used. Different cysteine conjugates were synthetized, by investigating different reducing agents at different concentration. It was important to setup the proper conditions to achieve the reduction of only the interchain disulphide bonds. The first reducing agent tested was DTT, at the concentration of 50 mM. The result was the total reduction of the antibody, so the reduction didn't occur only on the interchain disulphide bonds but involved also the intrachain ones. Then, TCEP was tested as reducing agent at increasing concentration. With 7.814 μM of TCEP the reduction didn't occur. With 1 mM of TCEP the reduction was partial. It was difficult to establish if only the interchain disulphide bonds and how many of them were reduced. With 3 mM and 10 mM of TCEP, the IgM was totally reduced. The reduction reaction could be followed by SDS-PAGE (Figure 3.4): it's clear that the best pattern was the one with TCEP 1 mM. The pattern of the IgM reduced with 50 mM of DTT was comparable to the one of a total reduction of an IgG (as shown in Figure 3.6).

After the reduction step the mixture was purified by SEC in FPLC. On the basis of the chromatograms, it was possible to confirm the results of SDS-PAGE: the profiles of TCEP 3 mM and TCEP 10 mM showed total reduction while the profile of TCEP 1 mM was the best one (Figure 3.5).

For this thesis project, the cysteine conjugates were realized using TCEP as the reducing agent, at the concentration of 7.814 uM (Cysteine 1) and 1 mM (Cysteine 2).

For the conjugation reaction, the mc-VC-PAB-MMAE linker-drug was used in excess with respect to the amount of free cysteines. The aim was to obtain a Drug-Antibody-Ratio of 20 (drug/IgM), in order to conjugate 4 molecules of drug per monomers (high loading conjugates). The equivalents of linker-drug per IgM were 6 for Cysteine 1 conjugate and 25 for Cysteine 2 conjugate. In particular, Cysteine 2 conjugate was realized with an excess of 1.25 of linker-drug/IgM. It's important

to work in excess in order to avoid possible loss of linker-drug during the conjugation step and to avoid incomplete cysteines conjugation.

After the purification in by SEC, the degree of conjugation was determined by UV, owing to the typical UV absorption of the drug. The determination took into consideration the values 280 / 248 nm before and after the conjugation step.

For the conjugate with 7.814 µM of TCEP and 6 Eq of Linker-Drug/IgM, the absorbance values obtained were:

- for the absorbance at 248 nm, 0.099 before conjugation and 0.129 after conjugation;
- for the absorbance 280 / 248 nm ratio, 2.44 before conjugation and 2.24 after conjugation.

These results showed sign of conjugation, because there was an increase in the absorbance at 248 nm (which was specific of the drug conjugated to the antibody) and a decrease in the absorbance at 280 / 248 nm ratio (which was a confirmation of the increase of the absorbance at 248 nm).

For the conjugate with 1 mM of TCEP and 25 Eq of Linker-Drug/IgM, the absorbance values obtained were:

- for the absorbance at 248 nm, 0.202 before conjugation and 0.220 after conjugation;
- for the absorbance 280/248 nm ratio, 2.37 before conjugation and 1.10 after conjugation.

Also, these values showed sign of conjugation, for the same reason of the previous conjugate.

Unfortunately, the exact DAR through a RP-HPLC determination of both conjugates couldn't be estimated owing the low drug loading.

The lysine conjugates were made using a different type of synthesis: the single step synthesis, where the reduction step was not required. The conjugation step was the only step necessary. For this conjugate, a different Linker-Drug was used: SuO-VC-PAB-MMAE, always in excess. This linker allowed the conjugation to ε amino group of the lysine of the IgM.

Also, for this type of conjugate different equivalents of Linker-Drug, with a variable excess, were used: 40 Equivalents (excess Linker-Drug / Eq IgM: 2), 60 Equivalents (excess Linker-Drug / Eq IgM: 3) and 70 Equivalents (excess Linker-Drug / Eq IgM: 3.5).

The conjugation step could be verified through a RP-HPLC measurement, that also allowed to obtain the effective DAR of the conjugates. The evaluation was done only for the lysine conjugate with 40 Equivalents of Linker-Drug. The result was a DAR of 15.5 (3.1 drug molecules per monomers). It was a good result, knowing that the conjugation of a large amount of drug-linker can be difficult.

For both cysteine conjugates and lysine conjugates, a SEC purification was done. The absorbance of the samples was evaluated at 280 nm.

For the cysteine conjugates, the purification was done after the reduction step to remove the excess of unreacted reducing agent and after conjugation to remove the excess of unreacted Linker-Drug and to exchange the buffer.

For the lysine conjugates, the purification was done only after conjugation, also to remove the excess of linker-drug not reacted and to exchange buffer.

Figure 3.6 and Figure 3.7 represents the purification of the cysteine conjugates: Figure 3.6 shows the chromatogram of the purification of the IgM post reduction instead Figure 3.7 shows the chromatogram of the purification of the final conjugates. The main peaks, (the protein reduced and the conjugates), eluted at around 8 mL. The intensity of the peak obviously changed because it depended on the amount of protein injected. What changed is the elution of the secondary peaks (peaks 2 on the chromatograms): the elution volume of the conjugates was higher than the elution volume of the IgM reduced. The secondary peaks could be associated to unreacted linker.

Figure 3.9 represents instead the purification of the lysine conjugates.

The purification showed that all 3 conjugates eluted with around the same elution volume and that the intensity of the peak corresponding to the unreacted Linker-Drug increased in line as the increment of the equivalents of Linker-Drug used. The elution volume of the conjugates was around 8 mL, equal to the volume of elution of the cysteine conjugates.

After the characterization of the conjugates, they were tested in vitro by the lab of Dr G. Toffoli at the "Centro di riferimento Oncologico" in Aviano (Italy). The conjugates tested were as follows: Lysine n.1 (Concentration= 0.7197 mg/ml; μg= 23.17), Lysine conjugate n.2 (Concentration= 0.1517 mg/ml; μg= 8.95) and Cysteine conjugate n.2 (Concentration= 0.177 mg/ml; μg= 205.32).

The aim was to study the activity of these conjugates on different cell lines. The cell lines tested were: BXPC3 (cell line expressing the receptor for GPC-1, Rec +) and Jurkat (cell line not expressing the receptor for GPC-1, Rec -). The final aim of my thesis project was to achieve an active targeting: the ADC should have been directed only on Rec + cells, because of the specific recognition of the receptor by the ADC.

The activity was evaluated at 48 h using a CellTiter Kit from Promega. The value considered was the IC_{50}, which represents the concentration of the conjugates that inhibits the 50 % of the metabolically active cells.

I expected a positive activity (on BXPC3 cell line) only for the lysine conjugates because the reduction, needed to obtain cysteine conjugates, was not necessary for these conjugates. The reduction of the disulphide bonds is the limiting factor of the synthesis: if it happens not only on the interchain disulphide bonds but also on the intrachain ones, it can lead to total disrupt of the conjugate. For this reason, I didn't expect a positive activity neither on the BXPC3 cell line nor on Jurkat cell line for the cysteine conjugates.

The absence of activity on the Jurkat cell line is a positive thing because it means that the ADC is not able to recognise a cell that doesn't express the specific receptor.

The first round of tests was done on both cell lines and were tested all the conjugate types. The concentrations of the conjugates employed were 100 nM; 10 nM; 1 nM; 0.1 nM; 0.01 nM. The IC_{50} concentration of the lysine conjugates on Jurkat cell line wasn't reached instead for the cysteine conjugate was equal to 6.391 nM. This can be seen on Figure 3.13. This is a negative thing because it means that there is activity also on the cells not expressing the receptor. The IC_{50} concentration of both conjugates on BXPC3 cell line wasn't reached, as shown in Figure 3.12.

These results can be confirmed by looking at the Figure 3.14 and Figure 3.15, where it was respectively described the cytotoxic effect of the lysine conjugates and cysteine conjugate on both cell lines.

The second round of tests was done only on BXPC3 cell line and were tested all the conjugate types. In this case, the concentrations exploited for the cysteine conjugate were: 500 nM; 50 nM; 5 nM; 0.5 nM and 0.05 nM instead for the lysine conjugates were: 200 nM; 20 nM; 2 nM; 0.2 nM and 0.02 nM.

From Figure 3.16, it's possible to see the cytotoxicity induced by the cysteine conjugate. The IC_{50} was reached at a concentration of the conjugate equals to 275.9 nM.

Figure 3.17 instead represents the cytotoxicity induced by the lysine conjugates. In this case the IC_{50} was not reached.

This test in fact was done as a confirmation of the results of the first round: in the first round only the cysteine conjugate was active (on both cell lines) and the same thing happened in the second round.

It's important to underline the fact that the cysteine conjugate induces more cytotoxicity on the Jurkat cell line than on the BXPC3 cell line (6.391 nM vs 275.9 nM). This means that the active targeting is not the only mechanism of recognition that occurs in the tumour cell, because of the activity on the Jurkat cell line, but the ADC suffers from unspecific recognition with cells.

Since the main activity was registered from the cysteine conjugate, another aspect that must be considered is the fact that, by reducing the IgM to obtain cysteine conjugates, its main function (CDC function) can be lost, because it's a function specific of an IgG. At this point, the effect related to the ADCs action is not mediated to the IgM itself but to the cytotoxic drug attached to it.

REFERENCES

1. *Martin GS. Cell signaling and cancer. Cancer Cell. 2003 Sep;4(3):167-74. doi: 10.1016/s1535-6108(03)00216-2. PMID: 14522250.*

2. *Cancer. (2022, February 3). https://www.who.int/news-room/fact-sheets/detail/cancer*

3. *Types of Cancer Treatment. (n.d.). National Cancer Institute. https://www.cancer.gov/about-cancer/treatment/types*

4. *Bertrand N, Wu J, Xu X, Kamaly N, Farokhzad OC. Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. Adv Drug Deliv Rev. 2014 Feb;66:2-25. doi: 10.1016/j.addr.2013.11.009. Epub 2013 Nov 22. PMID: 24270007; PMCID: PMC4219254.*

5. *Baah, S., Laws, M., & Rahman, K. M. (2021). Antibody–Drug Conjugates—A Tutorial Review. Molecules, 26(10), 2943. https://doi.org/10.3390/molecules26102943*

6. *Strebhardt, K. & Ullrich, A. Paul Ehrlich's magic bullet concept: 100 years of progress. Nature reviews. Cancer 8, 473–80 (6 2008).*

7. *Michinori Akaiwa, Julien Dugal-Tessier, Brian A. Mendelsohn, Antibody–Drug Conjugate Payloads; Study of Auristatin Derivatives, Chemical and Pharmaceu*

References

tical Bulletin, 2020.

8. *Chiu, M.L., Goulet, D.R., Teplyakov, A., & Gilliland, G.L. (2019). Antibody Structure and Function: The Basis for Engineering Therapeutics. Antibodies, 8.*

9. *Keyt BA, Baliga R, Sinclair AM, Carroll SF, Peterson MS. Structure, Function, and Therapeutic Use of IgM Antibodies. Antibodies (Basel). 2020 Oct13;9(4):53. doi: 10.3390/antib9040053. PMID: 33066119; PMCID:PMC7709107.*

10. *Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. The Adaptive Immune System. In Molecular Biology of the Cell, 4th ed.; Garland Science: New York, NY, USA, 2002;*

11. *Lu J, Jiang F, Lu A, Zhang G. Linkers Having a Crucial Role in Antibody-Drug Conjugates. Int J Mol Sci. 2016 Apr 14;17(4):561. doi: 10.3390/ijms17040561. PMID: 27089329; PMCID: PMC4849017.*

12. *Schrama D, Reisfeld RA, Becker JC. Antibody targeted drugs as cancer thera peutics. Nat Rev Drug Discov. 2006 Feb;5(2):147-59. doi: 10.1038/nrd1957. PMID: 16424916.*

13. *β-Galactosidase Cleavable Linkers - ADC / BOC Sciences. (n.d.). https://adc.bocsci.com/services/galactosidase-cleavable-linkers.html*

14. *Kang MS, Kong TWS, Khoo JYX, Loh TP. Recent developments in chemical conjugation strategies targeting native amino acids in proteins and their appli-*

cations in antibody-drug conjugates. Chem Sci. 2021 Oct 6;12(41):13613-13647. doi: 10.1039/d1sc02973h. PMID: 34760149; PMCID: PMC8549674.

15. *Zhou Q. Site-Specific Antibody Conjugation for ADC and Beyond. Biomedicines. 2017 Nov 9;5(4):64. doi: 10.3390/biomedicines5040064. PMID: 29120405; PMCID: PMC5744088.*

16. *Conilh L, Sadilkova L, Viricel W, Dumontet C. Payload diversification: a key step in the development of antibody-drug conjugates. J Hematol Oncol. 2023 Jan 17;16(1):3. doi: 10.1186/s13045-022-01397-y. PMID: 36650546; PMCID: PMC9847035.*

17. *Enzyme-mediated Modification and Conjugation - Creative Biolabs. (n.d.). https://www.creative-biolabs.com/adc/enzyme-mediated-modification-and-conjugation.htm*

18. *Staudacher AH, Brown MP. Antibody drug conjugates and bystander killing: is antigen-dependent internalisation required? Br J Cancer. 2017 Dec 5;117(12):1736-1742. doi: 10.1038/bjc.2017.367. Epub 2017 Oct 24. PMID: 29065110; PMCID: PMC5729478.*

19. *Pan J, Ho M. Role of glypican-1 in regulating multiple cellular signaling pathways. Am J Physiol Cell Physiol. 2021 Nov 1;321(5):C846-C858. doi: 10.1152/ajpcell.00290.2021. Epub 2021 Sep 22. PMID: 34550795; PMCID: PMC8616591.*

References

20. *Types of Tumors - Pancreatic Cancer | Johns Hopkins Pathology. (n.d.).*
    https://pathology.jhu.edu/pancreas/types-of-tumors

21. *Signs and Symptoms of Pancreatic Cancer. (n.d.). https://www.cancer.org/can*
    cer/pancreatic-cancer/detection-diagnosis-staging/signs-and-symptoms.html

22. *Pancreatic Cancer Treatment (Adult) (PDQ®)−Patient Version. (2020, Decem*
    ber 11). National Cancer Institute. https://www.cancer.gov/types/pancreatic/
    patient/pancreatic-treatment-pdq

23. *Sarantis P, Koustas E, Papadimitropoulou A, Papavassiliou AG, Karamouzis*
    MV. Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvi
    ronment and immunotherapy. World J Gastrointest Oncol. 2020 Feb
    15;12(2):173-181. doi: 10.4251/wjgo.v12.i2.173. PMID: 32104548; PMCID:
    PMC7031151.

24. *Foucher ED, Ghigo C, Chouaib S, Galon J, Iovanna J, Olive D. Pancreatic*
    Ductal Adenocarcinoma: A Strong Imbalance of Good and Bad Immunological
    Cops in the Tumor Microenvironment. Front Immunol. 2018 May 14;9:1044.
    doi: 10.3389/fimmu.2018.01044. PMID: 29868007; PMCID: PMC5960705.

25. *Cytiva. (n.d.). Size Exclusion Chromatography. https://www.cytivalifesciences.*
    com/en/us/solutions/protein-research/knowledge-center/protein-purification-
    methods/size-exclusion-chromatography

References

26. *Steward, K., PhD. (2022, May 9). Polyacrylamide Gel Electrophoresis, How It Works, Technique Variants and Its Applications. Analysis & Separations From Technology Networks. https://www.technologyne works.com/analysis/ar ticles/polyacrylamide-gel-electrophoresis-how-it-works-technique-variants-and-its-applications-359100*

27. *Walker JM. The bicinchoninic acid (BCA) assay for protein quantitation. Methods Mol Biol. 1994;32:5-8. doi: 10.1385/0-89603-268-X:5. PMID: 7951748.*

28. *Yao H, Jiang F, Lu A, Zhang G. Methods to Design and Synthesize Antibody-Drug Conjugates (ADCs). Int J Mol Sci. 2016 Feb 2;17(2):194. doi: 10.3390/ ijms17020194. PMID: 26848651; PMCID: PMC4783928.*

29. *CellTiter 96® AQueous One Solution Cell Proliferation Assay | MTS Assay | MTT Assay. (n.d.). https://nld.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter-96-aqueous-one-solution-cell-proli-feration-assay.*

30. *Munekage E, Serada S, Tsujii S, Yokota K, Kiuchi K, Tominaga K, Fujimoto M, Kanda M, Uemura S, Namikawa T, Nomura T, Murakami I, Hanazaki K, Naka T. A glypican-1-targeted antibody-drug conjugate exhibits potent tumor growth inhibition in glypican-1-positive pancreatic cancer and esophageal squamous cell carcinoma. Neoplasia. 2021 Sep;23(9):939-950. doi: 10.1016/j.neo.2021. 07.006. Epub 2021 Jul 28. PMID: 34332450; PMCID: PMC8340053.*

RINGRAZIAMENTI

La parte più difficile della stesura della tesi è sicuramente la parte dei ringraziamenti perché vorresti ringraziare tantissime persone ma lo spazio è quello che è.

Chi mi conosce sa quanto difficile sia per me esprimere affetto a voce e per questo magari, scrivendolo, mi risulterà più facile.

Inizio ringraziando la mia famiglia (mamma, papà e Antonio) per avermi aiutato in tutto durante questi sofferti anni. So a volte di essere stata un peso ma voi mi siete sempre stati accanto e mi avete sempre sostenuta, anche quando volevo mollare tutto. Avete sempre creduto in me e mi aveva spinta a puntare ancora più in alto.

Un ringraziamento speciale va a mia nonna Ilde perché, nonostante non avesse mai realmente capito cosa studiavo, si è sempre interessata e ha sempre cercato di essere presente, a modo suo. Per te io sarò sempre una farmacista, e va benissimo così. So di averti fatta dannare un bel po' ma alla fine ce l'ho fatta anche io a laurearmi.

Ringrazio poi i miei compagni di facoltà e compagni di università "acquisiti". Sapete che anche se non faccio tutti i vostri singoli nomi, ci sono e vi ricorderò per sempre. Anche senza volerlo, mi avete aiutata tantissimo e avete reso questi interminabili 5 (anzi 7) anni di università un po' meno pesanti. Con voi ho riso, scherzato, mangiato, bevuto e anche litigato. Ma con voi al mio fianco rifarei tutto da capo (sì, sono seria).

Ringrazio anche tutti i miei compagni di tesi perché hanno reso quei 9 mesi di tesi il percorso più bello (ma sofferto) di tutti gli anni di università. Ringrazio Antonella, Tommaso e Benedetta per avermi seguita e aiutata. Menzione speciale va fatta a Nicola B. (santo Nicola). Senza di te non sarei sopravvissuta (a Federica). Ti ringrazio perché riuscivi sempre a placare le mie mille incazzature quotidiane ma allo stesso tempo riuscivi a farmi ridere sempre. Ti ringrazio per non avermi mai fatta sentire a disagio, in nulla. Altra menzione speciale va fatta a Cecilia: grazie alle tue perle di armocromia, tutto il lab ora è in palette. Ringrazio

ovviamente il Professor Gianfranco Pasut per avermi dato la possibilità di svolgere la tesi presso il Suo laboratorio.

Ringraziamenti a parte vanno fatti ai miei coinquilini (e non), presenti e passati. Vi ringrazio per aver sopportato i miei scleri quotidiani, le mie lamentele e mille altri problemi. Grazie a voi ho imparato a mediare nelle cose (non ancora molto bene ma ci sto lavorando), ad ascoltare, ad aiutare.

So di essere stata spesso ostica, antipatica, incazzata ma il bene che vi voglio non è nemmeno lontanamente esprimibile a parole.

Voglio a tutti voi presenti un mondo di bene e, se non l'avessi già fatto altre mille volte durante questi ringraziamenti, vi ringrazio per esserci sempre stati. So di non essere una persona facile e per questo vi meritate un doppio ringraziamento. GRAZIE.