UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

NEW STRATEGIES FOR ANTIBODY DRUG DELIVERY

JOANA RITA GOMES OLIVEIRA

DISSERTAÇÃO ORIENTADA POR:

PROFESSOR DOUTOR JOÃO GONÇALVES

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“Ever tried.

Ever failed.

No matter.

Try again.

Fail again.

Fail better.”

Samuel Beckett

ABSTRACT

Antibody drug conjugates (ADCs) are growing as a new class of biopharmaceuticals that combines the specificity of monoclonal antibodies to deliver cytotoxic drugs selectively to a target antigen. In addition, monoclonal antibodies have demonstrated to have a significant importance in cancer treatment in contrast to classic chemotherapy which demonstrates limited selectivity against cancer cells resulting in a loss of efficacy. ADCs combine these two classes of drugs with their complementing characteristics, creating a highly selective and cytotoxic therapy.

Although ADCs have been under investigation for decades, with almost 120 total clinical trials, only two ADCs have been approved by FDA and EMA. The low cytotoxic drug potency and antigen selectively together with unstable linkers are the most frequent limitations identified in the ADC constructs. For this, understanding how each of the components of an ADC contributes to the efficacy and safety profile is essential to maximize clinical success. To this end, as data continue to emerge from the clinic, we will gain a better understanding of what changes are needed to improve the clinical activity of ADCs. Targeting the tumor microenvironment and include small antibody fragments are promising approaches for the development of future ADCs.

To overcome all the limitations presented in the previous ADCs, in the present work we demonstrate a proof-of-concept for the development of a new strategy for antibody drug delivery. For this, we engineered two bispecific VHH heterodimers composed by an anti-methotrexate VHH and an anti-CXCR4 VHH. To enhance the specificity to the tumor microenvironment, in one of the constructs (anti-MTX M1-CXCR4) sequence encoding a MMP-9 cleavage site was introduced to facilitate the release of cytotoxic drug in the target cells.

All constructions were successfully constructed, expressed and purify with high yields of purified soluble protein. For anti-MTX M1-CXCR4, ELISA demonstrated selective binding to methotrexate-serum albumin conjugate. Flow cytometry assay showed specific surface binding and internalization via CXCR4 receptor and an evident decrease in cell viability in the presence of recombinant proteins were assessed by MTT assay. Regarding anti-MTX M1-CXCR4, this protein induce cell death by a mechanism which is not completely understood. Preliminary assays showed that cell death is not dependent on the presence and release of MTX and induced toxicity in a concentration-

dependent manner in which a decrease in toxicity is a result of loss of function. In addition, cell death assays suggest that cells suffer necrotic death.

In conclusion, findings presented in this dissertation suggest that antibody-based constructs are promising therapeutic strategies to delivery cytotoxic drugs specifically to tumor microenvironment.

Keywords: Antibody drug conjugate (ADC); Antibody engineering; Cytotoxic drugs; Tumor microenvironment; Cancer

RESUMO

Os conjugados de anticorpo-fármaco (do inglês, *antibody-drug conjugates* (ADCs)) têm surgido como uma nova classe de biofármacos que combinam a especificidade dos anticorpos monoclonais com a entrega específica de fármacos citotóxicos.

Os anticorpos monoclonais desempenham um papel importante no tratamento de várias doenças, como o cancro e doenças autoimunes tornando-se assim, primeira linha de tratamento em algumas destas doenças. Além disso, a quimioterapia, que constitui a terapêutica clássica no caso de doenças oncológicas, caracteriza-se pela falta de selectividade contra as células cancerígenas levando a que estes fármacos apresentem uma janela terapêutica estreita, resultando na falta de eficácia. Os ADCs surgem como uma alternativa que combina as propriedades destas duas classes de fármacos, o que os torna altamente específicos e eficazes no tratamento de doenças oncológicas.

Apesar dos ADCs terem sido alvo de investigação durante as ultimas décadas, com aproximadamente 120 moléculas em ensaios clínicos, apenas dois ADCs foram aprovados pela agência norte-americana de produtos alimentares e de medicamentos (FDA) e pela agência europeia do medicamento (EMA). As limitações surgem sobretudo na falta de potência dos fármacos, na instabilidade da ligação do anticorpo ao fármaco e a baixa selectividade do anticorpo para o antigénio alvo.

Como os ADCs são constituídos por um anticorpo monoclonal, um fármaco citotóxico e um *linker* que une estes dois componentes é necessário perceber a contribuição de cada um dos componentes para aumentar a eficácia desta nova abordagem terapêutica. Com este propósito, à medida que se avança na investigação clínica, vai-se ganhando uma melhor compreensão sobre quais as mudanças necessárias a fazer para aumentar o sucesso dos ADCs. Bloquear antigénios com expressão aumentada no microambiente tumoral 0e substitutir os anticorpos monoclonais por pequenos derivados de anticorpos recombinantes têm surgido como estratégias promissoras para o desenvolvimento de novos ADCs.

De forma a ultrapassar as limitações apresentadas pelos ADCs desenvolvidos anteriormente, o objectivo proposto para este projeto científico foi o desenvolvimento e

prova de conceito de uma nova estratégia de ADCs. Para isso, foi construído um heterodímero biespecífico composto por dois anticorpos recombinantes (VHH, designado também como *nanobody*): um deles foi desenvolvido contra o metotrexato (MTX) e o outro contra receptor de quimiocinas CXCR4.

O MTX é um potente agente citotóxico usado no tratamento de várias doenças oncológicas e autoimunes. No entanto, o tratamento prolongado com MTX induz o aumento de efeitos adversos e de mecanismos resistência ao fármaco por parte das células cancerígenas. Por isso, o desenvolvimento de novos sistemas de entrega destas moléculas citotóxicas têm sido amplamente estudadas.

Por sua vez, o receptor de quimiocinas CXCR4 é um receptor transmembranar que apresenta a sua expressão aumentada em vários tipos de tumores sendo por isso considerado um alvo promissor para o desenvolvimento de novas terapêuticas.

Para validar a nossa estratégia, três VHH contra o MTX (anti-MTX VHH) foram sintetizados como monómeros. Em duas das construções foi ainda introduzida uma sequência de clivagem da MMP-9 (proteína da família das metaloproteinases da matriz) em dois locais diferentes na sequência do VHH de forma a facilitar a libertação do MTX para as células alvo. As três construções foram clonadas num vector de expressão bacteriano, expressas em *E.Coli* e purificadas posteriormente. Os resultados obtidos mostraram que apenas duas das construções, uma com o sitio de clivagem da MMP-9 e outra sem (anti-MTX M1 VHH e anti-MTX WT VHH, respectivamente), foram estavelmente produzidas em bactérias mantendo as propriedades naturais de ligação ao MTX.

Depois destes resultados preliminares, os heterodímeros biespecíficos foram então construídos tendo sido testada a posição do anti-MTX VHH (tanto a N-terminal como a C-terminal) com o objectivo de escolher a construção mais estável e solúvel. Como feito anteriormente, as construções foram clonadas num vector de expressão bacteriano e purificadas. Com base nos rendimentos obtidos nas purificações, as construções com o anti-MTX VHH a N-terminal foram selecionadas para os ensaios seguintes (anti-MTX WT-CXCR4 e anti-MTX M1-CXCR4).

Depois da expressão e purificação das proteínas recombinantes foi necessário confirmar que as propriedades de ligação ao MTX foram mantidas. Para isso, e uma vez que não é possível imobilizar o MTX a uma placa de ELISA foi necessário desenvolver uma estratégia em que o MTX foi acoplado à albumina (BSA-MTX) e usado como antigénio no ELISA. Os resultados demonstram que um dos heterodímeros biespecíficos (anti-MTX WT-CXCR4) e um dos monómeros (anti-MTX WT) ligam-se

especificamente ao MTX. No entanto, nas construções em que o anti-MTX M1 está presente verifica-se um aumento de interações não específicas com a BSA, usada como controlo

Em paralelo, um ensaio funcional feito apenas com os monómeros (anti-MTX WT VHH e anti-MTX M1 VHH) mostrou que na presença de MMP-9, o anticorpo é clivado e o MTX é libertado.

Uma vez que os heterodímeros são proteínas biespecíficas é necessário avaliar a especificidade dos dois domínios funcionais. Neste caso, e para avaliar a ligação ao CXCR4, foi realizado um ensaio de citometria de fluxo utilizando a linha celular Jurkat E6-1. Relativamente ao anti-MTX WT-CXCR4, os resultados sugerem que a proteína liga especificamente ao CXCR4 à superfície e é capaz de internalizar através deste receptor. Além disso, os resultados obtidos numa linha celular Jurkat CXCR4 negativa, evidenciam, mais uma vez, que a ligação e internalização desta proteína recombinantes é dependente do CXCR4.

No entanto, os resultados relativos ao anti-MTX M1-CXCR4 demonstram que esta construção induz morte celular.

Com o objectivo de tentar compreender o mecanismo pelo qual o anti-MTX M1-CXCR4 induz a morte celular, vários ensaios preliminares foram realizados. Os resultados sugerem que a morte celular não depende da presença e da libertação do MTX e que a toxicidade é dependente da concentração. Além disso, ensaios de morte celular sugerem que as células estão a morrer por um processo que induz necrose.

Com base em todos os resultados promissores obtidos anteriormente foi realizado um ensaio de citotoxicidade *in vitro* (MTT) de forma a avaliar a eficiência deste novo sistema de entrega de citotóxicos através de anticorpos. Os resultados mostram uma evidente diminuição na percentagem de células vivas para todas as contruções. No entanto, o anti-MTX M1-CXCR4 é o que apresenta uma maior redução na viabilidade celular.

Em paralelo, e uma vez que os fragmentos de anticorpos recombinantes exibem várias limitações farmacocinéticas, uma outra estratégia foi desenvolvida. Para isso, foi construída uma proteína biespecífica que contém um anticorpo monoclonal contra o receptor HER-2 (Trastuzumab, Herceptin®) acoplado ao anti-MTX VHH (anti-MTX WT e anti-MTX M1). Os resultados de ensaios de transfecção e W*estern Blot* sugerem que as proteínas recombinantes foram construídas e expressas com sucesso na linha celular HEK293T.

Como perspectivas futuras, é necessário realizar novos ensaios de forma a perceber qual o mecanismo que leva à morte celular na presença do anti-MTX M1-CXCR4. Além disso, os ensaios de citotoxicidade (MTT) terão que ser realizados com concentrações mais altas dos conjugados anticorpo-farmáco de forma a confirmar a eficácia *in vitro* destas construções. Além disso, é necessário desenvolver um método mais específico para a quantificação e purificação dos conjugados. Relativamente aos ensaios de ligação e internalização ao CXCR4, é necessário construir um heterodímero em que seja incluído um VHH irrelevante de forma a provar, por outra forma, que a ligação e internalização é dependente do receptor.

Em conclusão, os resultados obtidos demonstram que os heterodímeros de VHH biespecíficos desenvolvidos neste trabalho podem ser usados como uma nova estratégia promissora para a entrega de citotóxicos através de anticorpos surgindo assim como uma nova abordagem de ADCs.

Palavras-chave: Conjugados de anticorpo-fármaco (ADC); Engenharia de anticorpos; Fármacos citotóxicos; Microambiente tumoral; Cancro

ACKNOWLEDGEMENTS

Em primeiro lugar gostaria de agradecer ao Professor Doutor João Gonçalves, meu orientador, por me ter aceite no seu laboratório e por me ter proposto a realização deste projecto. Obrigada por ter confiado em mim e nas minhas capacidades e pelo incentivo e orientação ao longo deste ano.

Quero agradecer também aos meus colegas de laboratório, em especial à Catarina, à Ana Catarina, à Sofia Romano, ao Pedro, à Paula, ao Renato e à Rita. Obrigada por toda a paciência e por todos os minutos que perderam do vosso trabalho para me ajudarem com as dúvidas concretas e "existenciais" de todos os dias. Obrigada também pelos risos, pelos almoços, pelos lanches e pelas horas de conversa quando a vontade de trabalhar era ~~pouca~~ muita!

Não posso deixar de agradecer à Sofia Narciso por tudo o que passámos juntas neste último ano: aos dias no laboratório em que passávamos de dúvidas infinitas a certezas absolutas em segundos, aos dias intermináveis na biblioteca a escrever a tese, às batatas fritas que comemos juntas dia sim dia sim e principalmente por teres sido a minha companhia durante este ano. Obrigada!

Quero também agradecer aos meus amigos de sempre, principalmente à Catarina, à Joana, à Marta, à Márcia, à Helena e ao Mário. Obrigada por estarem sempre presentes quando eu mais preciso de vocês e por, cada uma à sua maneira, me ouvir, apoiar e por fazer parte dos melhores cafés, gins e risos ao longo destes meses.

Ao João, obrigada por tudo isto e por todos os dias. Obrigada por estares sempre do meu lado e pela paciência e compreensão nos dias mais difíceis. És o melhor!

Quero ainda agradecer, do fundo do coração, à minha família por me terem dado a oportunidade de ser o que sempre quis. À minha mãe, por seres um exemplo todos os dias, pela tua força, por nunca teres baixado os braços, por seres a minha melhor amiga e por todos os teus sábios conselhos. Não seria metade do que sou hoje sem ti. Ao meu pai, pelo teu lado racional, por me teres mostrado e ajudado a lidar com os problemas, por me ouvires e por teres sempre uma palavra de incentivo. Ao meu irmão, por tudo. Obrigada por me aturares e por me defenderes, obrigada por estares comigo desde sempre e por seres a pessoa que eu mais quero proteger no mundo.

Por fim, obrigada a todos os que, de uma forma ou de outra, tornaram isto possível.

ABBREVIATIONS

General

Aa Amino acid

Ab Antibody

ADCC Antibody-dependent cell-mediated cytotoxicity

ADME Adsorption, distribution, metabolism and excretion

Ag Antigen

CDC Complement-dependent cytotoxicity

CDR Complementary determining regions

C_H Constant heavy chain

CXCR4 CXC Chemokine Receptor 4

Fab Fragment antigen binding

Fc Fragment crystallizable

FDA Food and Drug Administration

HA Hemagglutinin tag

HER2 Human epidermal growth factor receptor 2 (also known as ErbB-2)

His Histidine tag

Ig Immunoglobulin

IgG Immunoglobulin G

mAb Monoclonal antibody

MW Molecular weight

scFv Single-chain variable fragment

sdAb Single-domain antibody

V_H Variable heavy chain

VHH Camel variable heavy chain

V_L Variable light chain

VNAR Shark heavy chain antibody

Reagents and Techniques

ABTS 2,2'-azino-bis(3ethylbenzothiazoline-6-sulphonic acid)

ELISA Enzyme-linked immunosorbent assay

HRP Horseradish peroxidase

IPTG Isopropyl-beta-D-thiogalactopyranoside

LB Luria broth medium

PBS Phosphate buffer saline

PCR Polymerase chain reaction

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Amino acids

A Alanine	**G** Glycine	**M** Methionine	**S** Serine
C Cysteine	**H** Histidine	**N** Asparagine	**T** Threonine
D Aspartic acid	**I** Isoleucine	**P** Proline	**Y** Tyrosine
E Glutamic acid	**K** Lysine	**Q** Glutamine	**V** Valine
F Phenylalanine	**L** Leucine	**R** Arginine	**W** Tryptophan

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INTRODUCTION

1.1. Antibodies

1.1.1. Antibodies – structure

Antibodies are an important class of proteins called immunoglobulins. Regarded as one of the most important defenses against disease, these proteins that exist naturally in human body are produced by the immune system in response to antigens - foreign substances that are capable of stimulating an immune response. There are five different classes of immunoglobulins - IgD, IgA, IgM, IgE and IgG - which differ in their ability to interact with the immune system [1]. Structural differences among the isotypes include differences in molecular weight and antigen binding sites [2]. IgG is the most abundant immunoglobulins in the blood (~80%) and from a biotechnology perspective, is the predominant format of therapeutic antibody [3].

Antibodies belonging to IgG class, a typical Y-shaped bivalent molecule, are organized in different function and structural domains. From a structure perspective, IgG is composed of two identical 25 kDa light chains (L) and two identical 50kDa heavy chains (H). The four polypeptide chains are covalently linked by disulfide bonds with a molecular mass of approximately 150 kDa. The heavy chains contain a variable domain (V_H) and three constant domains (C_H1, C_H2 and C_H3) and the light chains contain a variable domain (V_L) and a single constant domain (C_L) [4]

Functionally, IgG can be divided into two different units: the antigen-binding fragment (Fab) which is linked by a flexible region (hinge) to a constant fragment (Fc). The variable domains (V_H and V_L), in the amino terminal part of Fabs, determine the specificity, diversity and affinity of antigen binding. Each variable domain is composed by three regions of hypervariability where sequence variability is concentrated and loops are formed. These hypervariable regions are responsible for antigen recognition and because the antigen-binding site is complementary to structure of the epitope they are called the complementarity determining regions (CDRs 1, 2 and 3). The invariant regions of amino acids, among the CDRs, are very conserved segments designated framework region (FR) [4].

The Fc region corresponds to C_H2 and C_H3 portions of both heavy chains. It is distinct for each class of immunoglobulins, is glycosylated and contains sites for

interaction with effector molecules of the immune system [5].

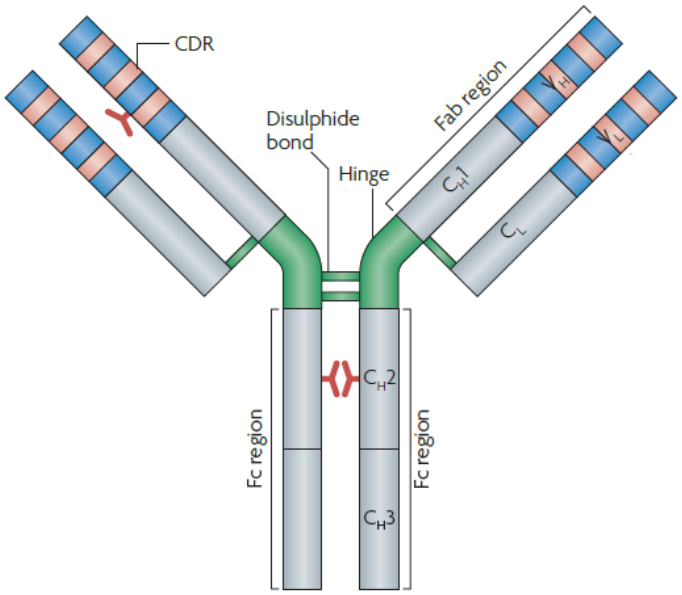

Figure 1 - Schematic representation of the structure of a conventional IgG antibody. Adapted from Beck, A. mAbs 6, 15-7 (2014) [80].

1.1.2. Antibody function

In human body, once a foreign substance has been recognized, the immune system recruits the participation of a range of cells and molecules to trigger a suitable response to eliminate or neutralize the foreign substance [6]. In this effector response, humoral immunity, also referred as antibody-mediated immunity, involves the effector functions of antibodies. They can act in two different manners: promote biological response that directly inactivate antigens or indirectly lead to their destruction through phagocytosis and complement activation [5].

The Fc portion of antibody recruits effector functions and it contains binding sites in the hinge region and the C_H2 domain for interaction with complement C1q molecule and/or interactions with Fc receptors (FcRs) [4]. Interactions with complement C1q molecule mediate complement dependent cytotoxicity (CDC) and interactions with FcyRs mediate antibody dependent cellular cytotoxicity (ADCC). In CDC, antibodies induce cell death by triggering the complement cascade at the cell surface. In ADCC, antibodies bind to antigens on the surface of target cells and the Fc domains engage Fc receptors (FcγRs)

on the surface of effector cells, such as macrophages and natural killer cells. These cells induce phagocytosis or lysis of the targeted cell [7].

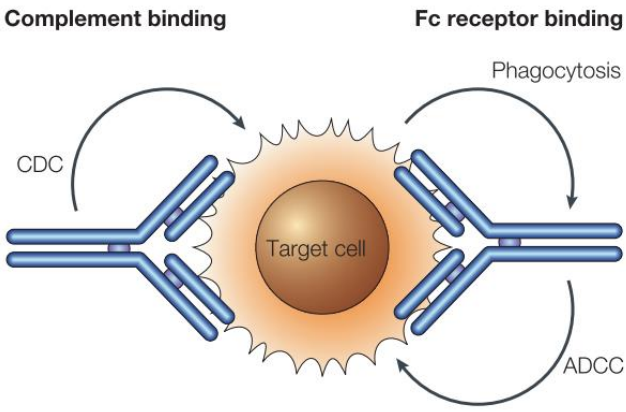

Figure 2 - ADCC and CDC: natural IgG effector functions. Adapted from Brekke, O. H. Nat. Rev. Drug Discov. **2,** 52–62 (2003) [8].

In case of antibodies used for therapeutic purposes, they have three recognize modes of action. They can block the action of specific molecules (like cytokines or growth factors) by preventing that they reach their target receptors in two different ways: by the antibody binding to the specific molecule itself or to its receptor [8].

The other mode of action is to target specific cells (e.g. tumor cells). It is a useful approach since antibodies can be engineered in order to carry effector payloads, such as toxins, cytotoxic drugs or enzymes, to the target cells. At last, they can function as signaling molecules since is it possible to target them to specific receptors and as a consequence, activate a specific cell population [8].

1.1.3. Development and production of therapeutic antibodies

Due to biological functions - high specificity and affinity to the target – antibodies started to be used as a potential tool in specific target therapies for the treatment of various diseases such as infection, cancer and autoimmune disorders [8].

The vast majority of antigens have multiple epitopes which leads to the proliferation and differentiation of B cell clones, each derived from a B cell that recognizes a specific

epitope. The resulting serum, known as polyclonal serum, is composed of a mixture of antibodies specific to a variety of epitopes [9].

For diagnostic, therapeutic or research uses, it is more desirable to use monoclonal antibodies derived from a single B cell clone and which recognize only one specific epitope [9]. To achieve this, several methods have been developed in order to produce monoclonal antibodies with predefined specificities.

In 1975, Köhler and Milstein described the mouse hybridoma technology [10] which is an important step for the emerged of monoclonal antibodies for therapeutic use. In this innovative technology, B cells secreting antibodies of one specificity were fused to a myeloid cell. The resulting hybrid cell is called a hybridoma, which exhibits immortality of a myeloid cell and the B cell properties of an antibody specific production [6].

Although this technology revolutionized the field of antibody research, early studies determined that monoclonal antibodies presented properties that could limit their clinical utility. First, B cells used for generation of hybridomas were from murine origin which implies that the human immune system recognizes murine monoclonal antibodies as foreign material and generated human anti-mouse antibodies (HAMAs) that result in their clearance from the body and consequently reducing their therapeutic benefit. In addition, murine monoclonal antibodies exhibit short serum half-lives and an incapacity to trigger human effector functions. To overcome these limitations, chimeric and humanized antibodies were developed in order to increase efficiency and reduce the immunogenicity of murine antibodies in human body [11].

The progress in antibody engineering allows manipulating antibodies in order to change them into more human variants while maintaining the properties of binding to the antigen. For this reason, monoclonal antibodies are now more suitable for use in therapeutic applications.

1.1.4. Recombinant antibody fragments

The clinically successful of therapeutic antibodies have been based on intact IgG however, the architecture of the IgG molecule facilitates the development of smaller antibody formats which are emerging as credible alternatives for a variety of therapeutic applications [3,12]. Because of their high molecular weight (~ 150 kDa), IgG antibodies present a poor penetration into tissues (e.g., solid tumors) and a slow clearance from the human body [4]. Antibody fragments have many advantages compared to immunoglobulins:

Fc-associated effects are avoid because there is no binding/activation of Fc receptor which reduces its immunogenicity; the rate of tissue penetration is increased; due to their low molecular weight, a range of therapeutic antibody fragments can be produced quickly in prokaryotes which reduce manufacturing issues (e.g., mammalian cell expression) [1,4].

In the recent years, with the help of antibody engineering, significant advances have been made in order to produce novel antibody fragments with specific characteristics. There is now a variety of new antibodies fragments which are in preclinical and clinical trials.

Fab (fragment antigen binding) consists of V_H-C_H and V_L-C_L linked by disulfide bonds. These antibody fragments with ~50 kDa maintain antigen-binding activity but since the Fc region is not present they are enable to stimulate the immune system functions which is an advantage in applications that include radioisotope or drug conjugation [13].

Other monovalent fragment is the single-chain variable fragment (scFv) (~25 kDa) that consists of V_H and V_L domains joined by a flexible linker. A peptide linker with around 10 to 25 amino acids in length and typically are rich in glycine for flexibility, as well as serine or threonine for solubility ($(Gly_4Ser)_3$) [11].

In general, ScFv tend to be seen as the smallest antibody fragments which preserve the antigen-binding sites. Nevertheless, single domain antibodies (sdAbs) which are composed by the V_H domain or V_L domain (~15 kDa) appear to maintain an important part of the original binding activity. Although this might at first appear to has a promising strategy, sdAbs have several limitations such as, low solubility and high tendency for aggregations. One way of overcoming this problems is identify and introduce mutations that reduce hydrophobic properties and select highly stable V_H or V_L domains from phage display libraries [11,14].

The camelids (camels and llamas) and cartilaginous fish (sharks) have heavy-chain only antibodies that are other promising alternative in the field of antibody fragments. The antigen binding site consists of a single unpaired variable domain (designated VHH for camelids and VNAR for sharks) which is able to recognize targets that are not easily recognized by monoclonal antibodies therapies. Other advantages include the manufacturing facility, improved tissue penetration, rapid blood clearance and high stability [11].

As a result, single domain antibodies are some of the most potent tools in therapy and diagnosis and because of this they are emerging as a new class of therapeutic molecules.

1.1.4.1. Single Domain Antibodies – VHH

As mentioned before, the field of recombinant antibody engineering has emerged as a powerful tool due to the fact that there is a strong interest in use antibodies for therapeutic applications.

The observation that camelids and sharks also produce unusual but functional antibodies composed only for heavy chains has been increased the interest in using single domain antibodies instead of intact IgG. These heavy chain antibodies lack light chains and the antigen binding site is formed by a single domain that is linked to the Fc-domain [15].

Based on sequence and structure analysis of the VHH domains, several conserved structural characteristics have been found. Structurally, VHHs have four framework regions (FRs) which are responsible for the core structure of the antibody domain. In addition, they have three CDRs that are involved in antigen-binding activity. Although the whole structure is very similar to V_H domain, a point mutation in the framework 2 (FR-2) region leads to that a more hydrophilic structure [1,15]. In addition, VHHs scaffold is composed by two α-sheeted structures. Regarding complementary-determining regions and in comparison with V_H domains, CDR1 is extended at N-terminal by four amino acids that participate in antigen recognition. The CDR2 loop structures differ from the canonical loops structures that are defined for V_Hs, although the principal residues are conserved. The CDR3 is longer and enables recognition of cavities or hidden epitopes on the surface of the antigens [16].

VHHs have several advantages for biotechnological applications which include their small size (~15 kDa), high solubility, stability, specificity and affinity, ease of cloning and thermal and chemical resistance. Moreover, they can be easily produced in bacterial system which is a very cost-efficient process [17].

In general, single domain antibodies enables subsequent molecular manipulation. For example, depending on the application, it is useful to engineer monovalent fragments into multivalent antibody fragments to increase avidity or to produce bispecific antibody fragments that can bind to different antigens at the same time. These antibody fragments, known as diabodies, can be produced using linkers between the V_H and V_L domains, although this often results in protein aggregation. In contrast, VHHs can be produced more easily in these formats since they facilitate the inclusion of more flexible linkers which is

important for the binding of multivalent antigens. As an example, several trivalent bispecific VHHs have been successfully produced [15,18].

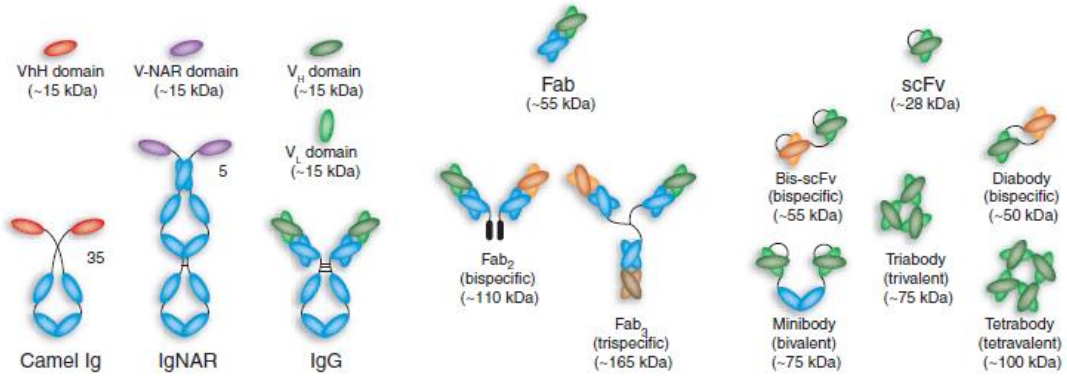

Figure 3 - Schematic representation of different antibody fragments. Adapted from Holliger, P. Nat. Biotechnol. **23,** 1126–36 (2005) [1].

1.1.5. Pharmacokinetics of intact antibodies versus fragments

Pharmacokinetics is defined as the study of the time course of drug adsorption, distribution, metabolism and excretion (ADME) [19]. The pharmacokinetics characteristics are influenced by diverse factors including size, charge, shape, hydrophilicity, proteolytic degradation and interaction with molecules and cells [20].

Therapeutic proteins, or small molecules drugs, have well-defined properties allowing the development of drug delivery systems and choosing the right therapeutic dosage. Regarding distribution volume of proteins, it depends on their molecular weight, physicochemical properties and their dependency on active transport processes. In most cases, therapeutic proteins have high molecular weight and large sizes which means that their apparent volume of distribution is typically small [21].

As mentioned before, engineering antibodies exhibit high affinity, specificity, reduced immunogenicity and low cross-reactivity. Because of all these specific characteristics, a major consideration in protein engineering is to improve the pharmacokinetics properties: appropriate dosing leading to optimal bioavailability, uptake, distribution and clearance in targeted and non-targeted tissues, resulting in optimal pharmacodynamics [22].

One of the most important parameter in pharmacokinetics and biodistribution of therapeutic proteins is the protein size. IgG molecules are too large for rapid tumor penetration, high target retention and rapid blood clearance which leads to high serum levels and associated toxicities [23]. Conversely, single domain fragments, as VHHs, have a fast tissue penetration and exhibit a rapid blood clearance because of their small size of about 15 kDa. Since they can rapidly pass the renal filter (cut off of about 60 kDa), several strategies have been developed in order to prolong circulation of these recombinant antibodies in the blood and thus improve administration and pharmacodynamics properties [1,23].

One approach to increasing the half-life of antibodies is the PEGylation of antibodies and antibody fragments, which reduces their immunogenicity by chemical coupling of polyethylene glycol (PEG) to amino groups of the antibody [11,23]

Significant advances have been made in the past decade towards the discovery, optimization and therapeutic application of antibodies in a wide range of diseases. In addition, antibody fragments have specific characteristics and properties which made them a powerful therapeutic and diagnostic agents as well as monoclonal antibodies.

1.2. Antibody drug conjugates

In the recent years, antibodies have become an important drug class: more than 60 antibodies are approved for human therapy and about 240 are currently in preclinical and clinical trials for several diseases such as, inflammation and autoimmunity, cancer, infectious and cardiovascular diseases [24].

Although all recent approvals, oncology has been a principal area of focus for monoclonal antibodies-based therapies mainly because a variety of overexpressed antigens in cancer cells compared with normal cells have been identified [25]. Because of their high specificity and ability to bind target antigens, antibodies have long been a significant tool in basic research. However, many challenges have to be overcome in order to produce more therapeutic antibodies with clinical application. As a result, significant attention has turned in enhancing antibody activity by conjugate cytotoxic drugs to them, generating antibody drug conjugates (ADCs) capable of drug delivery in a site-selective manner [26].

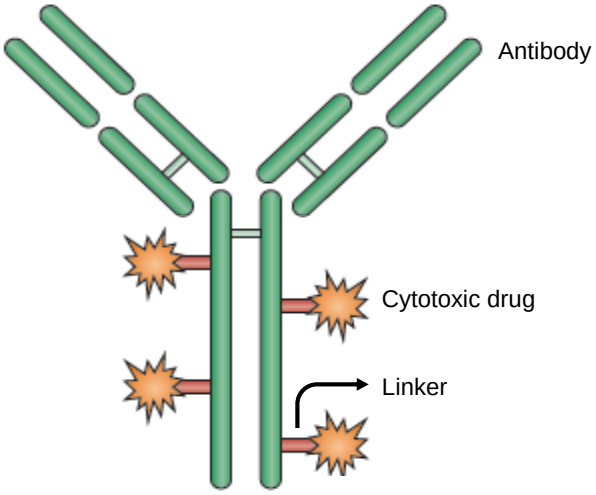

Figure 4 - Antibody drug conjugate structure which includes the antibody, the linker and the cytotoxic drug. Adapted from Zolot, R. S. Nat. Rev. Drug Discov. **12,** 259–60 (2013) [81].

1.2.1. History of ADCs

The traditional treatment of cancer includes chemotherapies that use drugs which target quickly dividing cancer cells. These drugs include the folate and purine analogs which are DNA damaging agents and microtubule polymerization inhibitors [27]. One of the limitations about these drugs is the severe side effects since they target both normal and cancer cells. Consequently, the therapeutic index for these drugs is small and have a narrow therapeutic window [28]. In order to overcome this limitation in drug development, ADCs appeared as a new strategy. The promise of ADCs was that they could selectively deliver cytotoxic drugs into target cells, a concept first described by Paul Ehrlich as “Magic Bullets” in the early 1900s [29]**.** Knowledge gained from the initial development of ADCs has led to an improved understanding of ADCs function and clinical applications.

1.2.1. ADCs function and mechanism of action

The rationale behind an ADC strategy is to ideally deliver the cytotoxic drug only to cells expressing the target antigens. This process starts when the antibody binds to its

antigen. Regarding target antigen, it must be located in the cell surface in order to be reached by the antibody. Established ADC binding, the whole antigen-ADC complex is internalized through receptor-mediated endocytosis [30] which is a process that starts when a ligand binds a cell surface receptor and triggers a cascade of events that includes the formation of early endosomes and the transported to late endosomes and lysosomes [31].

In lysosomes, the cytotoxic drug is released from the antibody and can interfere with different cellular mechanisms resulting in cell death [30]**.** Nearby cancer cells may also be killed if the cytotoxic drug is released into the tumor microenvironment in a process known as the bystander effect [32].

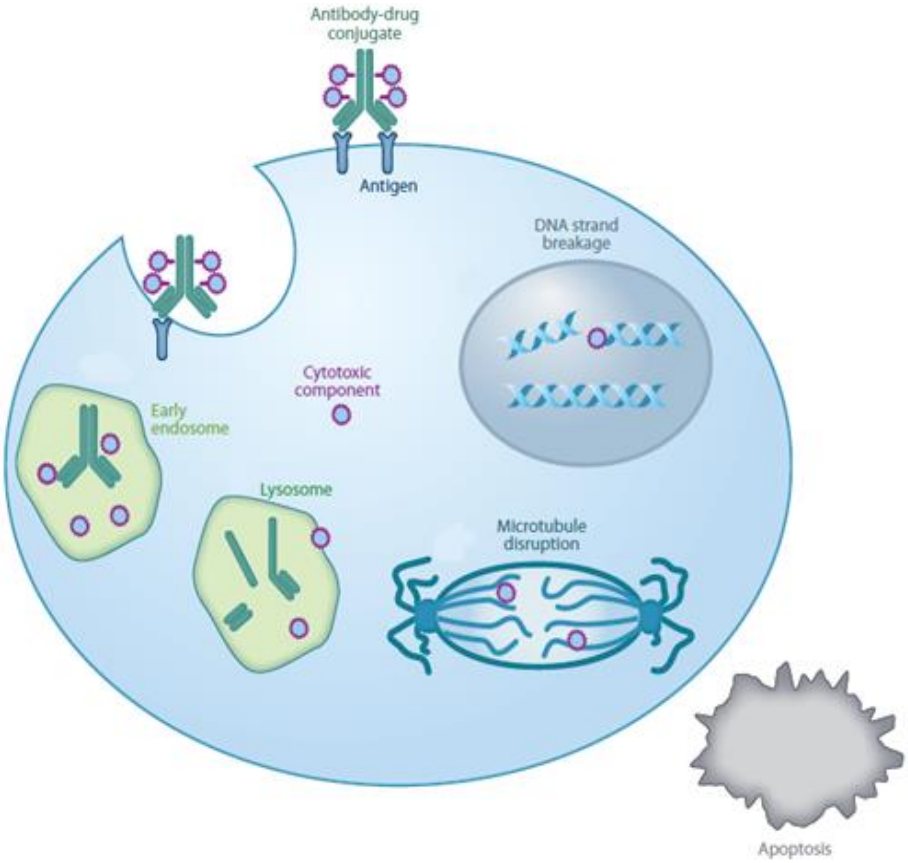

Figure 5 - Generalized mechanism of action of antibody-drug conjugates (ADCs). Adapted from Sievers, E. L. Annu. Rev. Med. **64,** 15–29 (2013) [30].

To determine the success of an ADC, the anatomy of the conjugate which include antigen target, cytotoxic drug, antibody and linker, are important key areas of research.

1.2.2. Anatomy of ADCs

Over the past years, extensive research has been done in order to transform the early-generation ADCs into effective therapeutic strategies. For this, several changes which include a better understanding of target selection, advances in antibody engineering and improvements in linker and cytotoxic drug conjugation strategies were made [33]. A brief description of each ADC components is presented in the following sections.

1.2.2.1. Antibody selection

The antibody is a crucial factor that affects ADC success. For this reason, monoclonal antibodies have been engineered to be extremely specific in antigen binding for a various diseases. Majority of approved therapeutic monoclonal antibodies are based on IgG 1 isotype. However, initial studies showed a heterogeneous distribution of these monoclonal antibodies in solid tumors. These results reveal that high affinity antibodies have low tissue penetration capacity [34]. The non-uniform distribution in tumor tissue are called “binding site barrier” which is the result of high non-specific protein binding within tumors [35]. As an alternative, and as mention in the previous section, conventional IgG could be engineering in small antibody fragments.

The ideal antibody in an ADC would have the following characteristics: it induces receptor-mediated endocytosis; it is engineered against a surface antigen that is overexpressed on the target cells; it produces a low immune response in humans; the cytotoxic drug conjugation does not affect the antibody stability, internalization and binding and finally, it has a long half-life to allow significant accumulation in target cells [36].

1.2.2.2. Cytotoxic drug

The use of an optimal payload, which includes potent small molecules with low specificity, contributes to the success of an ADC [36]. In addition, drugs must have a suitable functional group for conjugation and need to be stable under physiological conditions [27].

In general, there are two categories of payloads for conjugation to monoclonal antibody: radionuclides and cytotoxic drugs. Regarding radionuclides, they emit a

radiation that penetrates into the target cells and induce a lethal response with no or minimal damage to the normal cells [37].

The cytotoxic drugs include high potency synthetic or natural small molecules. Early ADC development focused on the use of readily available and clinically approved drugs such doxorubicin, methotrexate, mitomycin, fluorouracil and vinca alkaloids [36].

Regarding methotrexate (MTX), it is one of the most studied and effective chemotherapy agent and immune system suppressant used for the treatment of solid tumors, autoimmune and hematologic diseases [38]. MTX is a folic acid analog and act as a cancer chemotherapeutic agent by inhibiting dihydrofolate reductase (DHFR) with high affinity, resulting in depletion of tetrahydrofolates that are needed for the synthesis of purines and pyrimidines. Consequently, the synthesis of DNA, RNA and other metabolic reactions are interrupted [38,39]. In addition, long-term treatment and clinical studies have shown that the curative effect of MTX was reduced because it led to toxic dose-related side effects and because of the drug resistance of the tumor cells. Based on this, MTX was already used in a variety of delivery systems [40]

The limiting clinical activity exhibited by initial ADCs led to the development of a new generation of ADCs employing more potent cytotoxic drugs which are extremely toxic to use in an untargeted manner but have sufficient potency to be used as a payload [30]. With this in mind, the cytotoxic drugs currently being used to construct ADCs belong to two categories: microtubule inhibitors (e.g. auristatins and maytansinoids) and DNA-damaging agents (e.g. anthracyclines, calicheamicins, duocarmycins, and pyrrolobenzodiazepines) [27]. Efficacy and toxicity should be balanced in order to successfully develop new ADCs.

1.2.2.3. Linker

The linker that connects the cytotoxic drug to the antibody is an important determinant of ADC activity and should be stable in circulating blood and enable rapid release of cytotoxic drug inside target cells [41].

ADC linkers are divided into two important categories: cleavable and non-cleavable linkers. The cleavable linkers can be divided into acid-labile linkers which are designed to be stable at pH levels that was found in the blood, but become unstable and degrade at low pH environment; a protease-cleavable linkers are also designed to be stable in blood, but rapidly release free drug inside lysosomes upon cleavage by lysosomal enzymes and

a third type of linker used contains a disulfide linkage which take advantage of the high level of intracellular reduced glutathione to release free drug inside the cell [27].

Non-cleavable linkers exhibit high stability in the blood, but are exclusively dependent on receptor-mediated endocytosis to release active drug and kill target cells. In addition, they may not release drug in extracellular space and consequently, they are not able to kill neighboring cells [27,42].

The chemical method for the attachment a drug to antibody continues unchanged. So, site-specific conjugation, in which a known number of linker-drugs are consistently conjugated to defined sites, is one way to overcome the challenges of the conventional methods [43]. Heterogeneity is minimized and ADC properties are maintained from batch to batch. [27].

There are three methods for site-specific conjugation:

1. To avoid the problem of heterogeneity, cysteine residues can be engineered in a proper site in which cysteine substitution does not alter the structure or function of the protein. THIOMAB conjugates are an example of this approach [43].

2. The use of enzymes with high specificity for a given substrate is another strategy for use in site-specific conjugation [44].

3. The incorporation of an unnatural amino acid, such as acetylphenylalanine instead of alanine. [45,46].

The main characteristic, common to all linkers, is to release the cytotoxic drug specifically in the target cells in order to control the toxicity of the highly potent drugs used to construct ADCs.

1.2.2.4. Target Selection

The nature of the target antigen affects the safety and efficacy of therapeutic antibodies. The ideal tumor antigen must be located at cell surface to allow ADC binding. The antigens expressed by cancer cells have revealed a vast range of targets that are overexpressed, mutated or selectively expressed compared with normal cells. Another

important characteristic of the tumor antigen is the capacity to internalize upon ADC binding. If ADCC or CDC is the required mechanism of action it is desirable that the antigen–antibody complex should not be rapidly internalized in order to maximize the availability of the Fc region to trigger an immune effector. In contrast, good internalization is preferable for antibodies that deliver cytotoxic drugs into the cancer cell [47].

Considerable effort has been made in order to identify new antigen targets that are appropriate for antibody-based therapies. For this purpose, serological, genomic, proteomic and bioinformatics databases have been used to identify antigens that are overexpressed in tumor cell populations or that are linked to gene mutations identified as driving cancer cell proliferation [12,47].

1.3. Cancer

1.3.1. Carcinogenesis

Cancer is a leading cause of death worldwide with approximately 14 million new cases and 8 million cancer related deaths per year. According to World Health Organization, cancer is characterized by the uncontrolled growth of cells, which can invade and spread to distant sites of body [48]. Over the past years, research in this area has revealed that cancer is a disease that involves dynamic changes in the genome, with several mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function [49,50]. These initial discoveries at molecular level indicate that carcinogenesis is a multistep process which involves genetic alterations that drive the progressive transformation of normal cells into highly malignant derivate cells [49,50].

Therefore, it is now well established that genotype and phenotype of cancer cells differs from normal cells and in 2000, Douglas Hanahan and Robert A. Weinberg identified six hallmarks alterations in cancer cell physiology: self-sufficiency in growth signals; insensitivity to growth-inhibitory signals (antigrowth); evasion of programmed cell death (apoptosis); limitless replicative potential; sustained angiogenesis and tissues invasion and metastasis [50].

Although all malignant characteristics, metastasis is the principal cause of cancer morbidity and mortality and is the term used to describe the dissemination of cancer cells from primary tumor to surrounding tissues and to distant organs [51] .

The invasion and metastasis process has been characterized as a sequence of events which is known as invasion-metastasis cascade. The first step started with local invasion, then intravasation by malignant cells through the basal membrane into a blood or lympatic vessels. After that, cancer cells leave the blood vessels by a process known as extravasation and proliferate at a secondary site forming small nodules of cancer cells (micrometastases) that can become macroscopic tumors. This last step is called colonization [51,52].

The mechanisms mentioned above continue to provide a solid foundation for understanding the biology of cancer. However, progress in cancer research made over the past decade has demonstrated that tumors are more than insular masses of proliferating cancer cells: they are complex tissues composed of multiple cell types that participate in interactions with one another [52].

1.3.2. Tumor microenvironment

The malignant characteristics of cancer cells also depended on an important interaction between cancer cells and their local environment. The capacity to interact and change its surroundings is an important characteristic by which cancer cells are able to develop mechanisms essential for tumor growth and metastatic dissemination [53].

The tumor microenvironment describes the non-cancerous cells present in the tumor. These include fibroblasts, immune cells and endothelial cells. It also includes the proteins produced by all the cells present in the tumor that support the growth of cancer cells [54].

Understanding the molecular mechanisms of this interaction between cancer cells and their tumor microenvironment represents one of the principal challenges in cancer research.

1.3.3. Matrix Metalloproteinases

It is now well established that the development, invasion and metastasis of cancer recruits the tumor microenvironment for the participation in essential mechanisms during the process [55].

Extracellular proteinases, such as the matrix metalloproteinases (MMPs), mediate many of the alterations in the tumor microenvironment during tumor progression. MMPs are a family of zinc-dependent endopeptidases that degrade several components of the extracellular matrix (ECM), including collagen, laminin, fibronectin, elastin and proteoglycans. In addition, they play an important role in various physiological processes including organ development and tissue remodeling, in the regulation of inflammatory processes [56].

Among the many MMPs that have been identified, gelatinases, especially MMP-2 (gelatinase A) and MMP-9 (gelatinase B), have been reported as having an increased expression in many human tumors [57].

The discovery that specific MMPs are implicated as overexpressed targets in a particular disease, several strategies have been developed in order to inhibit or use specific MMPs sequence to cleave prodrugs and release free drug in a particular tissue [58]. As an example, GPQGIAGQ is a well-studied MMP-sensitive peptide derived from collagen type I which sensitive to a variety of collagenase including MMP-1, MMP-2, MMP-3, and MMP-9 [59].

Bearing this in mind, the next generation of ADCs may be able to development a novel formulation that is activated specifically in the tumor microenvironment to enhance anti-tumor activity.

1.3.4. C-X-C chemokine receptor type 4 – CXCR4

As previously mentioned, signaling from the tumor microenvironment has significant implications in the maintenance and progression of hematopoietic and epithelial cancers. Stromal cells, which represent the majority of non-neoplastic cells present in the tumor microenvironment, secrete constitutively the chemokine stromal cell-derived factor-1 (SDF-1/CXCL12). CXCL12 secretion attracts cancer cells by stimulation of CXCR4 receptor, which is expressed by hematopoietic and epithelial cancer cells [60].

Chemokine receptors belong to the family of G protein coupled receptors (GPCR) which form the biggest group of signal transducing transmembrane proteins [61]. In addition, these receptors mediate chemotaxis of cells towards a gradient of chemokines [62]. Moreover, CXCR4 receptor is rapidly internalized and re-expressed after ligand binding by a process dependent on phosphorylation [63]. Structurally, CXCR4 has a seven-transmembrane structure with seven helical regions connected by six extramembrane loops [62].

CXCR4 is involved in several mechanisms which promote tumor progression: is crucial for metastatic spread to organs where CXCL12 is expressed; CXCL12 itself can stimulate survival and growth of cancer cells and promote tumor angiogenesis by attract endothelial cells to the tumor microenvironment [60].

A decade ago, researchers demonstrated the overexpression of CXCR4 in human breast cell lines and primary and metastatic breast tumors [64]. Today, CXCR4 overexpression is known in more than 20 human tumor types and, consequently, increased expression of CXCR4 is a negative predictor of survival and a strong predictor of tumor relapse in patients [65]. It has also been established that CXCL12 is highly expressed in local, regional, and distant metastatic sites, such as lymph nodes, bone marrow, lung, and liver, thus suggesting that the CXCR4/CXCL12 axis plays a major role in regulating the destination of most tumor cell metastases [66].

Promising results in preclinical tumor models indicate that CXCR4 antagonists may have antitumor activity [60]. Collectively, these observations reveal that CXCR4 is an important molecule involved in the progression of a variety of different tumors and therefore can be considered a very attractive strategy as a tumor target for ADCs.

1.4. Preclinical and clinical development of ADCs

In the past decade, considerable advances in the treatment of cancer have been developed using selective small molecules that specifically target oncogenic drivers. These therapies have shown significant results in diseases that are well defined by a single genetic mutation; however, they have a limited activity in more complex diseases. In addition, conventional chemotherapy induces adverse effects because it kill both cancer and normal cells. As a result, combining the potent cytotoxic effects of chemotherapeutics with a targeted approach would improve the therapeutic index of conventional

chemotherapies [33]. With this in mind, ADCs represent a promising therapeutic approach for the treatment of cancer.

Current innovations in monoclonal antibodies engineering, linker technologies and the identification of highly potent cytotoxic drugs lead to entry of more than 30 ADCs into clinical development and two drugs into the market to treat various hematological and solid tumors: Ado-trastuzumab emtansine (T-DM1, Kadcyla®, Roche-Genentec) and Brentuximab vedotin (BV, SGN-35/Adcentris®, Seattle Genetics).

Ado-trastuzumab emtansine is an ADC composed of trastuzumab, a humanized monoclonal antibody targeting the oncogene HER2, linked to lysine residues with the maytansinoid DM1 by the noncleavable SMCC thioether linker. Once the ADC is internalized and directed to lysosomes, the antibody is completely degraded with lead to intracellular release of the lysine-linked maytansinoid resulting in cell cycle arrest and consequently, cell death. This ADC receiving approval by the FDA in 2013 for treatment of $HER2^{+}$metastatic breast cancer [30].

Brentuximab vedotin is a next-generation ADC. It consists of an anti-CD30 monoclonal antibody linked to the antimitotic agent monomethyl auristatin E (MMAE) via a cleavable valine citruline peptide linker. The ADC binds to the CD30 antigen and undergoes rapid internalization and proteolysis, leading to the efficient release of MMAE inside the target cells. It was approval for the treatment of two indications: patients with Hodgkin lymphoma (HL) and patients with systemic anaplastic large cell lymphoma (ALCL). [30].

Without exception, ADCs in the clinic are based on conventional IgG however, all the knowledge based on the previous ADCs research are generally transferable to antibody fragments which have demonstrated great potential in therapeutic and diagnostic applications. In addition, nanobody-drug conjugate are desirable formats for the development the next generation of ADCs.

In two independent studies [67,68], a nanobody-drug conjugate were successfully engineering fused a VHH antibody fragment to a cytotoxic drug or a bacterial toxin via chemical conjugation. In both cases, nanobodies are very convenient tools for delivering toxic cargos to cancer cells.

As mentioned before, signals from the tumor microenvironment may make significant contributions to the progression of hematopoietic and epithelial tumors.

The discovery of CXCR4 functions as a co-receptor for X4 HIV-1 viruses involving during the course of HIV-1 infection generated a high interest in the development of small

molecular CXCR4 receptor antagonists for the treatment of HIV. With the emergence of the physiologic functions of CXCR4, other potential applications of CXCR4 antagonists are becoming apparent. As noted, the CXCR4-CXCL12 axis may play a central role in the spread and progression of many different types of tumors. Molecules that target this receptor or its ligand could mobilize tumor cells from their specific microenvironments and make tumor cells more accessible to conventional therapy [60].

Currently, CXCR4 antagonists (AMD3100 and ALX40-4C) as well as CXCR4 antibodies (e.g. BMS-936564) are being evaluated in preclinical and clinical studies for the treatment of neoplastic or autoimmune disorders. In any case, both CXCR4 antagonists appear to have activity in various cancers. The key role of CXCR4 in the tumor microenvironment context suggests that CXCR4 therapeutic approaches may become relevant in the near future [60].

In 2014, Kularatne *et al* describe the development of an anti-CXCR4 IgG chemically conjugated to auristatin which demonstrate *in vitro* and *in vivo* efficacy against a human osteosarcoma cell line implanted in the tibia of a mouse and then derived as metastasized cells from the lung [66].

In conclusion, taking advantage of the synergies between ADC componentes contributes to increase efficacy and safety profile of this therapeutic approach in order to maximize the clinical success of an antibody drug delivery system.

1.5. Aims

Monoclonal antibodies have demonstrated considerable utility in the clinical treatment of a variety of diseases, in particular for cancer. As a result, there is a considerable interest in improved antibody activity by attached cytotoxic drugs to them, generating antibody drug conjugates for the site specific delivery of drugs. Significant progresses have been made in ADC research; however, they continue to present several limitations with only two ADC with market approval.

With this in mind, new strategies for the development of ADCs are required to delivery payloads to specific tumor cells. For this, the aim of the present work is to engineered monoclonal antibodies into more suitable fragments in order to target overexpressed components in tumors cells and delivery cytotoxic drugs directly in the tumor microenvironment developing a new generation of ADCs.

To achieve this goal, we will develop two different bispecific VHH heterodimers composed by an anti-methotrexate VHH attached to an anti-CXCR4 VHH. In one of the bispecific proteins, it will be include a sequence encoding a MMP-9 cleavage site in the anti-methotrexate VHH framework in order to facilitate the release of the cytotoxic drug in the target cells.

Thus, the overall goals of this thesis are the following:

1) Construction of bispecific VHH heterodimers;

2) Optimization of the expression and purification conditions for bispecific VHH heterodimers;

3) Characterization of recombinant proteins binding properties against methotrexate and CXCR4 receptor.

4) Evaluation of *in vitro* efficacy of antibody drug conjugate

1.5.1. Outlined strategy

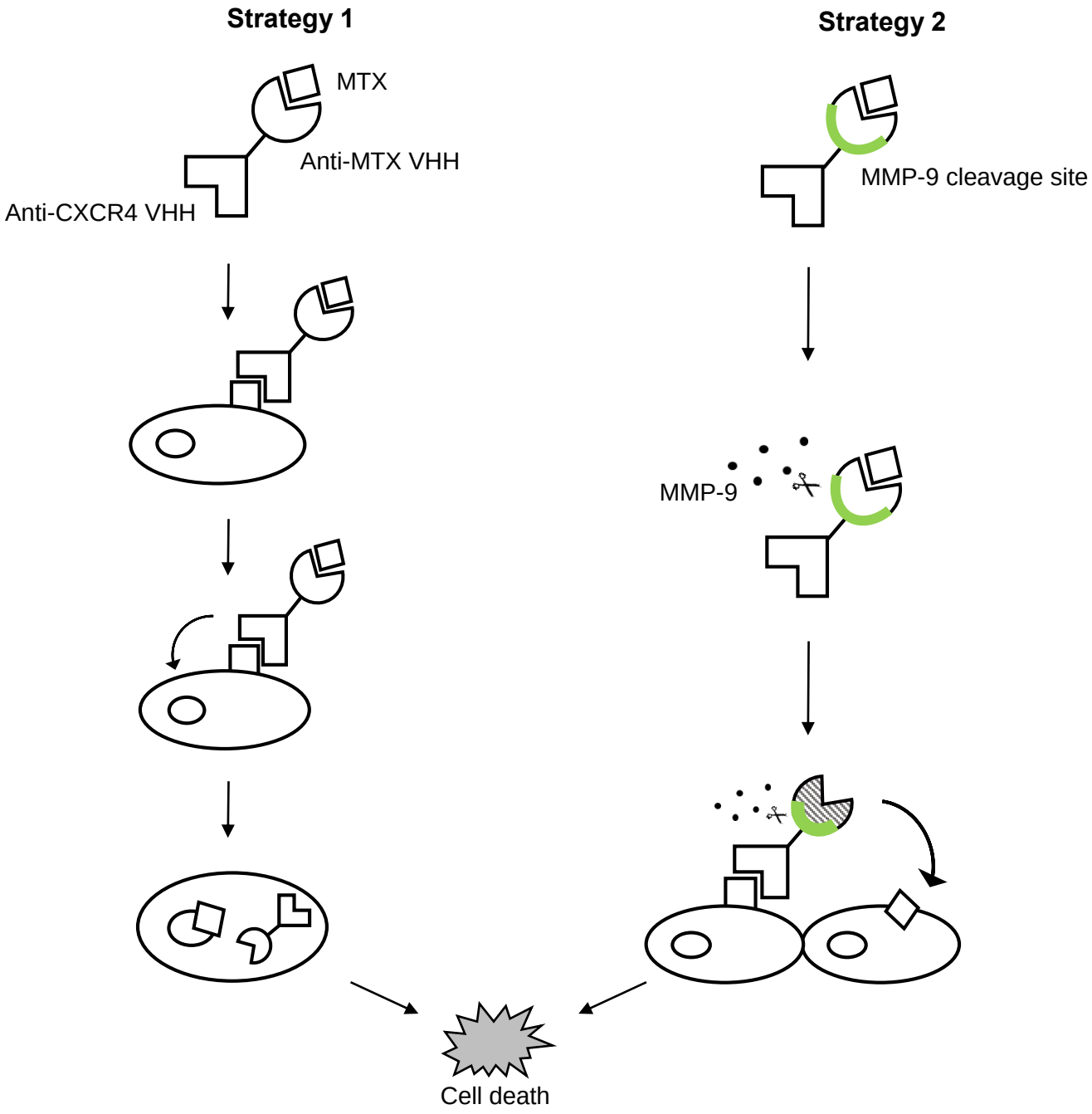

Figure 6 – Schematic representation of the strategy proposed for VHH heterodimers mechanism of action.

Since our strategy includes two different VHH heterodimers (with and without MMP-9 cleavage site), it is expected that we have two different mechanisms of action. In relation to strategy 1, with VHH heterodimer that does not have the MMP-9 cleavage site, it is expected that this protein binds to CXCR4 receptor, internalizes and efficiently release the cytotoxic drug inside the target cell. Regarding strategy 2, with VHH heterodimer that includes MMP-9 cleavage site in the anti-MTX VHH framework, the MMP-9 produced by extracellular matrix should cleave the VHH antibody fragment and release the cytotoxic drug in the tumor microenvironment inducing cell death by a mechanism that does not require ADC internalization.

MATERIALS AND METHODS

2.1. Cloning of recombinant proteins

2.1.1. Anti-MTX VHH wild type, mutant 1 and mutant 2

DNA encoding anti-MTX VHH wild type (WT) [69], anti-MTX VHH mutant 1 (M1) and anti-MTX mutant 2 (M2) were synthesized by Invitrogen adding a sequence encoding peptide tags for purification (His_8) and detection (FLAG). To the anti-MTX VHH mutant 1 and 2 were also include a sequence encoding a MMP-9 cleavage site (GPQGIAGQ) in two different positions of the VHH antibody framework (insertion sequence position: Gln 170 for mutant 1 and Asp 305 for mutant 2).

A fragment encoding the anti-MTX VHH WT, M1 and M2 were amplified by PCR with specific primers (Table 4, Annexes) and subcloned into the bacterial expression vector pET-21a(+) using the NheI and XhoI restriction enzyme.

2.1.2. Anti-MTX WT-CXCR4 VHH

To construct the anti-MTX WT-CXCR4 VHH bispecific heterodimer, a DNA fragment comprising the entire anti-MTX WT VHH was generated by PCR with specific primers (Table 4, Annexes), adding NheI and SacI restriction sites at the fragment 5' and 3' ends, respectively. The same procedure was done to the anti-CXCR4 VHH [70] using different primers (Table 4, Annexes), adding SacI and XhoI restriction sites at the fragment 5' and 3' ends, respectively.

The resulting PCR fragments were gel purified (Zymo Research, USA), digest with NheI/SacI and SacI/XhoI restriction enzymes (anti-MTX VHH and anti-CXCR4, respectively) and cloned into the appropriately cut pET-21a(+) vector. A short GS linker (SGGGGS) was used to link the anti-MTX VHH and anti-CXCR4 VHH.

2.1.3. Anti-MTX M1-CXCR4 VHH

The protocol used for the cloning of the anti-MTX M1-CXCR4 VHH bispecifc heterodimer is identical to the protocol described in 2.1.2. PCR was performed with specific primers (Table 4, Annexes).

2.1.4. Anti-CXCR4-MTX WT VHH

The protocol used for the cloning of the anti-CXCR4-MTX WT VHH bispecifc heterodimer is identical to the protocol described in 2.1.2. PCR was performed with specific primers (Table 4, Annexes).

2.1.5. Anti-CXCR4-MTX M1 VHH

The protocol used for the cloning of the anti-CXCR4-MTX M1 VHH bispecifc heterodimer is identical to the protocol described in 2.1.2. PCR was performed with specific primers (Table 4, Annexes).

All constructs were carried out by DNA digestion with enzymes from Thermo Fisher Scientific (UK). T4 DNA Ligase (Thermo Fisher Scientific, UK) was used in all vector-insert ligations. Polymerase Chain Reaction (PCR) was performed in Doppio Thermocycler (VWR International, USA) using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, UK), according to the manufacture's instruction. All primers used for PCR reactions are presented in table 3 in annexes. All PCR conditions programs used are presented in table 4 in annexes. Clones were screened by PCR reaction and resolved by agarose gel electrophoresis. Positive clones sequence was confirmed by DNA standard sequencing (GATC Biotech, Germany).

2.2. Expression and purification of proteins

To express and purify all the recombinant proteins, positive clones were transformed into *E. Coli* strain BL21 (DE3) or into SHuffle® T7 Competent *E. coli* (only for anti-MTX WT-CXCR4 VHH).

2.2.1. Anti-MTX WT VHH

For the expression of anti-MTX WT, 500 mL of LB medium containing 100 µg/mL ampicillin (Nzytech, Portugal) was inoculated with 5 mL of overnight culture of bacterial cells and grown to exponential phase (A_{600}=0.6-0.9) at 37 °C. Expression was induced

by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific, UK) and growth during 16h at 16°C. Cells were harvested by centrifugation (12 000 × *g*, for 5 min at 4 °C), resuspended in 12.5 mL of equilibration buffer (10 mM Tris, 250 mM NaCl, 20 mM imidazole, pH 8) and lysed by sonication. Cell debris were removed by centrifugation (12 000 × *g*, for 60 min at 4 °C) and the supernatant was filtered through a 0.45 µm syringe filter (Sarstedt, Germany).

All chromatographic steps were performed at 4°C. The anti-MTX WT extract was purified by nickel chelate affinity chromatography in a His GraviTrap™ column (GE Healthcare, UK). After a washing step (10 mM Tris, 250 mM NaCl, 50 mM imidazole, pH 8), bound proteins were eluted with 300 mM imidazole and buffer exchange to 10 mM Tris, 250 mM NaCl, pH 8 was performed using Disposable PD-10 Desalting Columns (GE Healthcare, UK) according to the manufacturer's instructions.

2.2.2. Anti-MTX M1 VHH

For the expression of anti-MTX M1, 500mL of LB medium containing 100 µg/mL ampicillin (Nzytech, Portugal) was inoculated with 5 mL of overnight culture of bacterial cells and grown to exponential phase (A_{600}=0.8-0.9) at 37 °C. Expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific, UK) and growth during 4h at 37°C.

After 4h expression, cells were harvested by centrifugation (12 000 × *g*, for 5 min at 4 °C), resuspended in 20 mL buffer B (50 mM HEPES, 1 M NaCl, 10 mM imidazole, 5 mM $CaCl_2$, 1 mM β-mercaptoethanol, 2 M urea, pH 8) and lysed by sonication for 30 min. Cell pellet/ insoluble fraction was collected by centrifugation (12 000 × *g*, for 30 min at 4 °C), resuspended in 20 mL buffer B (50 mM HEPES, 1 M NaCl, 10 mM imidazole, 5 mM $CaCl_2$, 1 mM β-mercaptoethanol, 2 M urea, pH 8) and lysed by sonication for 30 min. Cell pellet/insoluble fraction was recollected by centrifugation (12 000 × *g*, for 30 min at 4 °C), resuspended in 50 mL buffer C (50 mM HEPES, 1 M NaCl, 10 mM imidazole, 5 mM $CaCl_2$, 1 mM β-mercaptoethanol, 6 M urea, pH 8).

The protein pellet resuspended in buffer C is subjected to a denaturation/solubilization step overnight at 4ºC in a vertical rotator (Stuart Rotator, Dynalab). The solubilized protein was submitted to centrifugation (12 000 × *g*, for 60 min at 4 °C) for removal of remaining cell debris and insolubilized protein. The solubilized protein solution was filtered through a 0.45 µm syringe filter.

All chromatographic steps were performed at 4 °C. The recombinant proteins were purified by nickel chelate affinity chromatography in a His GraviTrap™ column (GE Healthcare, UK) according to the manufacturer's instructions for purification under denaturing conditions. Bound proteins were eluted with 50 mM HEPES, 1 M NaCl, 500 mM imidazole, 5 mM $CaCl_2$, 1 mM β-mercaptoethanol, 6 M urea, pH 8 and buffer exchange to 10 mM Tris, 250 mM NaCl, pH 8 was performed using Disposable PD-10 Desalting Columns (GE Healthcare, UK) according to the manufacturer's instructions.

The experimental protocol was adapted from Cunha-Santos et al. [71].

2.2.3. Anti-MTX M2 VHH

The protocol used for the expression and purification of anti-MTX M2 VHH is identical to the protocol described in 2.2.2.

2.2.4. Anti-MTX WT-CXCR4 VHH

For the expression of anti-MTX WT-CXCR4 VHH, 500mL of LB medium containing 100 µg/mL ampicillin (Nzytech, Portugal) was inoculated with 5 mL of overnight culture of bacterial cells and grown to exponential phase (A_{600}=0.4-0.6) at 30°C. Expression was induced by the addition of 0.4 mM IPTG (Thermo Fisher Scientific, UK) and growth during 4h at 30°C. Cells were harvested by centrifugation (12 000 × *g*, for 5 min at 4°C), resuspended in 12.5 mL equilibration buffer (10 mM HEPES, 250 mM NaCl, 20 mM imidazol, pH=7.4) and lysed by sonication. Cell debris were removed by centrifugation (12 000 × *g*, for 60 min at 4°C) and the supernatant was filtered through a 0.45 µm syringe filter (Sarstedt, Germany).

All chromatographic steps were performed at 4°C. The protein extract was purified by nickel chelate affinity chromatography in a His GraviTrap™ column (GE Healthcare, UK). After a washing step (10 mM HEPES, 250 mM NaCl, 20 mM imidazol, pH=7.4), bound proteins were eluted with 300 mM imidazole and buffer exchange to 10 mM HEPES, 250 mM NaCl, pH=7.4 was performed using Disposable PD-10 Desalting Columns (GE Healthcare, UK) according to the manufacturer's instructions.

2.2.5. Anti-MTX M1-CXCR4 VHH

The protocol used for the expression and purification of anti-MTX M1-CXCR4 VHH is identical to the protocol described in 2.2.2.

2.2.6. Anti-CXCR4-WT VHH

The protocol used for the expression and purification of anti-MTX-CXCR4 VHH M1 is identical to the protocol described in 2.2.4.

2.2.7. Anti-CXCR4-M1 VHH

The protocol used for the expression and purification of anti-CXCR4-MTX M1 is identical to the protocol described in 2.2.2.

Protein purity was analyzed by SDS-PAGE and protein concentration was determined by Bradford method according to the manufacturer's instructions (Bio-Rad, USA).

2.3. Coomassie staining

Protein separation was performed according to the method of Laemmli in 15% polyacrylamide gels (SDS-PAGE). Following electrophoresis, the gel was placed in staining solution (40% methanol, 10% acetic acid, 0,025% Coomasie Brilliant Blue). The gel was incubated for 20 min to 1h in the staining solution. The gel was destained with several changes of destain solution (30% methanol, 10% acetic acid) until the background is transparent. All staining/destaining steps were done on a rotary shaker with gentle mixing.

2.4. Western Blot

Protein separation was performed according to the method of Laemmli in 15% polyacrylamide gels (SDS-PAGE). Once separated, the proteins were electrotransferred into a nitrocellulose membrane (GE Healthcare, UK). Membrane was blocked with a 5% milk-TBS 0.1% Tween20 for 1 h and proteins were detected as follow. Anti-MTX WT and M1 were detected using a HRP-conjugated anti-FLAG-tag (Sigma-Aldrich, USA), diluted 1:8000 in 5% milk-TBS 0.1% Tween20 for 60 min at room temperature with agitation. Anti-MTX WT-CXCR4 and M1 were detected using a HRP-conjugated anti-HA-tag (Sigma-Aldrich, USA) diluted 1:5000 in 5% milk-TBS 0.1% Tween20 for 60 min at room temperature with agitation. Membrane was washed 5 times with TBS 0.1% Tween20 solution. Antibody detection was made with ImmobilonTM Western Chemiluminescent HRP substrate (Millipore, USA). Membranes were incubated with HRP substrate for 5 min at room temperature and then revealed in a chemiluminescence film Amersham Hyperfilm^{TM} ECL (GE Healthcare, UK).

2.5. BSA-MTX conjugation

BSA-MTX conjugate was prepared via carbodiimide linkage between carboxylic acid groups of each drug molecules and the lysines, which have primary amines that can react with the amine reactive intermediate compound; the solution obtained was purified after dialysis.

The conjugation process of MTX to BSA is described as follows: Briefly, MTX was dissolved in phosphate buffered saline (PBS, pH 7.4) and ‘activated’ with 5-fold excess of N-(3-dimethylaminopropyl)-N´- ethylcarbodiimide hydrochloride and 1- fold excess of N-hydroxysulfosuccinimide for 2 h at room temperature. To each activated drug molecule was then added 10 mg/mL of BSA and left reacting during 8-10 hours, starting all the reactions in moles ratio of 75:1 drug to BSA. Excess of drug molecules and by-products were removed from conjugated protein using 10-kDa-molecular-sizecutoff (MWCO) membrane (Merck Millipore).

This protocol was kindly performed by Rita Acúrcio from João Gonçalves lab.

2.6. Enzyme-linked immunosorbent assay (ELISA) to evaluate the bispecific heterodimers VHH to methotrexate

BSA-MTX (2 µg/well) was adsorbed onto 96 well flat bottom, high binding non-sterile, polystyrene ELISA plates (Corning, USA) overnight at 4°C and the remaining binding sites were blocked with 3% BSA (Sigma-Aldrich, USA) in PBS. After 1 h of blocking, several dilutions of the purified samples were incubated for 1h at 37°C. The plates were washed with tween 20 (0.05% in PBS) and detection was performed with HRP-conjugated anti-FLAG-tag or HRP-conjugated anti-HA-tag (Sigma-Aldrich, USA) diluted 1:1000 in 1% BSA (in PBS). The plates were then washed with PBS and developed with an HRP substrate, ABTS solution (citric acid (pH 4) with 0.2% H_2O_2) (Calbiochem, Germany). Absorbance was measured at 405/490 nm in a microplate reader (Bio-Rad, USA)

2.7. Determination of specific cleavage by metalloproteases

To determine if anti-MTX M1 (which has a MMP-9 cleavage site) could specifically release methotrexate in the presence of MMP-9, anti-MTX WT and M1 (310 mM) were incubated with methotrexate fluorescein isothiocyanate conjugate (MTX-FITC) (Life Technologies, USA) at 37°C for 1 h. Afterwards, the antibody-drug conjugates were exposed to nickel-charged resin at RT for 1 h. MMP-9 (Sino Biological, China) was added (500 ng) to the previous mixture at 37ºC for 1h and the fluorescent intensity (490 nm of excitation maximum and 525 nm of emission maximum) was measured in Tecan Infinite M200 plate reader (Switzerland).

2.8. Cell culture conditions

Jurkat E6-1 T-cells obtained through the NIH AIDS Research and Reference Reagent Program were cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 0.25 µg/mL Amphotericin B (RPMI-10). Cells were grown in tissue culture flasks (25cm^3) (Sarstedt, Germany) at 37ºC with 5% CO_2.

Jurkat CXCR4 negative cell line was constructed and kindly provided by C. Cunha-Santos (João Gonçalves laboratory, unpublished results). In addition, this cell line was cultured in the same conditions as described above.

Human embryonic kidney (HEK) 293T (ATCC, VA, USA) cell line was cultivated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL Penicillin, 100µg/mL Streptomycin, and 0.25 µg/mL Amphotericin B (DMEM-10). Cells were grown throughout 70-80% of confluence in tissue culture flasks (75 cm^3) (Sarstedt, Germany), at 37°C with 5% CO_2.

All cell culture media and reagents, otherwise indicated, were from Lonza (Switzerland).

2.9. Flow cytometry assay to evaluate VHH heterodimers binding to CXCR4

For each assay condition, Jurkat E6-1 T cells and Jurkat CXCR4 negative cell line were seeded at $2x10^5$ per well in 96-well plates (Sarstedt, Germany). Recombinant proteins (2 µM) were previous incubated with methotrexate-fluorescein isothiocyanate conjugate (MTX-FITC) (Life Technologies, USA) (1 µM) at 37°C for 1 h. Since CXCR4 receptor internalized it was necessary to evaluate surface binding and internalization. For this, the antibody drug conjugate was incubated with cells at 4°C (no internalization, only surface binding) or 37°C (internalization) for 2 h. Following 2 h of incubation, cells which were incubated at 37°C were washed twice with trypsin (to eliminate antibody drug conjugate surface binding) and then with PBS. Cells which were incubated at 4°C were washed twice with PBS. MTX-FITC, anti-CXCR4 VHH and anti-MTX VHH (WT and M1) were used as control.

To evaluate toxicity from recombinant proteins, serial dilutions of anti-MTX WT/M1-CXCR4 were incubated with cells in the same conditions as explained above.

Bispecific VHH heterodimers binding to CXCR4 were detected by excitation at 488nm and detection at 525 nm. Flow cytometry analysis was performed in a Guava® easyCyte HT (Millipore, USA), by acquirement of 5000-gated events from each sample. Data were analyzed using FlowJo software (Tree Star, USA).

This experimental protocol was designed and optimized by C. Cunha-Santos (João Gonçalves laboratory, unpublished results).

2.10. Assessment of cell viability in the presence of the recombinant proteins

Recombinant proteins were incubated with methotrexate (Sigma-Aldrich, USA) for 1 h and purified by repeated washing with PBS using an Amicon concentrator with 10 kDa MWCO (Millipore, USA). The amount of conjugate concentration was determined spectrophotometrically measuring the absorbance at 280 nm in Nanodrop ND-1000 (Thermo Fisher Scientific, USA) and calculated using the calculated molar exctinction coefficients value of each protein (ε VHH heterodimers = 27515 M-1 cm-1 and ε VHH monomers = 34045 M-1 cm-1).

Jurkat E6-1 T cells were seeded at $2x10^5$ per well in 24-well plates. The maximum concentration recovered from amicon purification was serial diluted and free MTX was adding in the same concentration for 24, 48 and 72 h at 37ºC. In each time point, 100 µL of cells were collected from well and washed with PBS followed by addition of 100 µL of MTT solution (0.5 mg/mL in PBS). Afterwards, the cells were reincubated for 3-4 h to facilitate the formation of formazan crystals. The excess solution was then aspirated carefully, and MTT formazan crystals were dissolved in 100 µL of DMSO (Sigma-Aldrich, USA). The absorbance at 490 nm was measured in microplate reader (Bio-Rad, USA).

2.11. Cloning and expression of Trastuzumab plus anti-MTX WT/M1

2.11.1. Trastuzumab plus anti-MTX WT

To construct Trastuzumab plus anti-MTX WT a fragment encoding anti-MTX VHH WT was amplified by PCR with specific primers (Table 4, Annexes). PCR fragment was gel purified and subcloned into the mammalian expression vector pCEP4 using HindIII restriction enzyme. pCEP4 vector was previously cloned with a fragment encoding Trastuzumab, which was kindly provided by Dr. Christophn Rader [72,73].

2.11.2. Trastuzumab plus anti-MTX M1

The protocol used for the expression and purification of Trastuzumab plus anti-MTX M1 is identical to the protocol described in 11.1. PCR was performed with specific primers (Table 4, Annexes).

2.11.3. Transfections

HEK293T cells were transfected by the calcium phosphate method [74]. 5 x 10^5 cells were seeded in each well of 6-well plates (34,7 mm Ø) (Sarstedt, Germany). Twenty-four hours after, cells were transfected with 5 µg of total DNA according to protocol. Cell medium was changed the next day and 48 hours after transfection cells were harvested.

2.11.4. Immunoprecipitation and western blot analysis

Transfected cells were washed twice with phosphate-buffer saline (PBS) solution and lysed with a RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % Sodium Deoxycholate, 0.1 % SDS, H_2O) supplemented with complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Germany) on ice. Thirty minutes after, cells were centrifuged (14 000 x g, 30 min, 4˚C) and cell supernatant was recovered.

To precipitation of immune complexes, both supernatants (from cell transfection plates and lysates) were exposed to native protein A sepharose 4 fast flow (GE Healthcare, UK). Following 1 h of incubation at 4ºC, the complexes were centrifuged (12 000 x g, 20 seconds) and pellets were washed three times with PBS and once with wash buffer (50 mM Tris, pH 8). The final pellets were suspended in sample buffer (1% SDS, 100 mM DTT, 50 mM Tris, pH 7.5).

Total protein of each sample was resolved in a 12% SDS-polyacrylamide gel. Proteins were electrotransferred into a nitrocellulose membrane. Membrane was blocked with a 5 % milk-TBS 0.1% Tween20 solution for 1 h and proteins were detected using a HRP-conjugated anti-HA-tag diluted 1:5000 in 5 % milk-TBS 0.1% Tween20 for 1 h at RT with agitation. Membrane was washed 5 times with TBS 0.1% Tween20. Antibody detection was made with ImmobilonTM Western Chemiluminescent HRP substrate (Millipore, USA). Membrane were incubated with HRP substrate for 5 min at RT and then revealed in a chemiluminescence film Amersham HyperfilmTM ECL (GE Healthcare, UK).

RESULTS

3.1. Construction, expression and purification of bispecific VHH heterodimers

For the development of our therapeutic antibodies, bispecific VHH heterodimers were constructed in order to promote the release of cytotoxic drugs in the tumor microenvironment and its delivery to cancer cells.

The recombinant proteins were generated by fusing an anti-MTX VHH [69] to an anti-CXCR4 VHH [70] including a histidine tag (His8) followed by a hemagglutinin tag (HA) in C-terminal. To take advantage of the particular properties in the tumor microenvironment, in two of the anti-MTX VHH constructs (anti-MTX M1 and anti-MTX M2) a MMP-9 cleavage site was introduced in the VHH antibody framework (Gln 170 for mutant 1 and Asp 305 for mutant 2). The heterodimeric fusion proteins present 303 amino acids residues with a calculated molecular weight of ~35 kDa. Anti-MTX WT, M1 and M2 VHHs were also constructed as monomers and used as control in the future assays, in these cases a FLAG tag was including at C-terminal and used for detection. These proteins present 105 amino acids residues with a calculated molecular weight of ~16 kDa. All constructs are shown on figure 7.

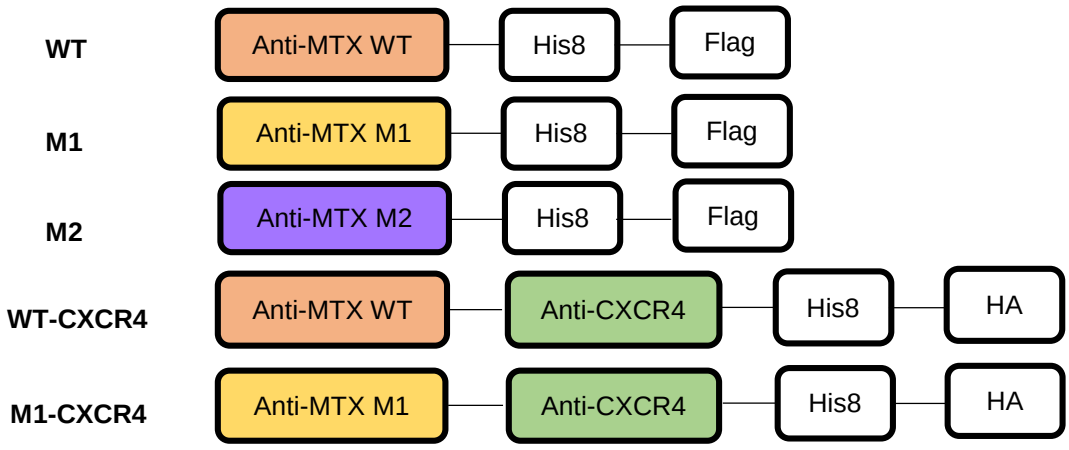

Figure 7 - Schematic representation of VHH monomers and bispecific heterodimers constructions.

From the expression assays, it was possible to verify that all constructs containing anti-MTX WT were expressed and purified from the soluble fraction. In contrast, all constructs presenting anti-MTX M1 were expressed and purified from insoluble fraction. Regarding anti-MTX M2, this construct presented a residual protein expression in both fractions. The expression conditions of all recombinant proteins are depicted in Table 1.

Table 1 - Optimal expression conditions for the recombinant proteins.

Protein	*E.Coli* strain	Expression time (h)	Induction IPTG (mM)	Temperature (ºC)
WT	BL21 (DE3)	16	1	16
M1	BL21 (DE3)	16	1	37
WT-CXCR4	Shuffle T7	4	0.4	30
M1-CXCR4	BL21 (DE3)	16	1	37

After optimization of the expression conditions (table 1), it was necessary to purify the proteins. All constructs were purified by Immobilized Metal Affinity Chromatography (IMAC) with a nickel column and after that submitted to buffer exchange (see section 2.2. from materials and methods). SDS-PAGE and Western Blot (data not shown) results showed a single protein band with the expected molecular weights for the recombinant proteins under reducing conditions (figure 8).

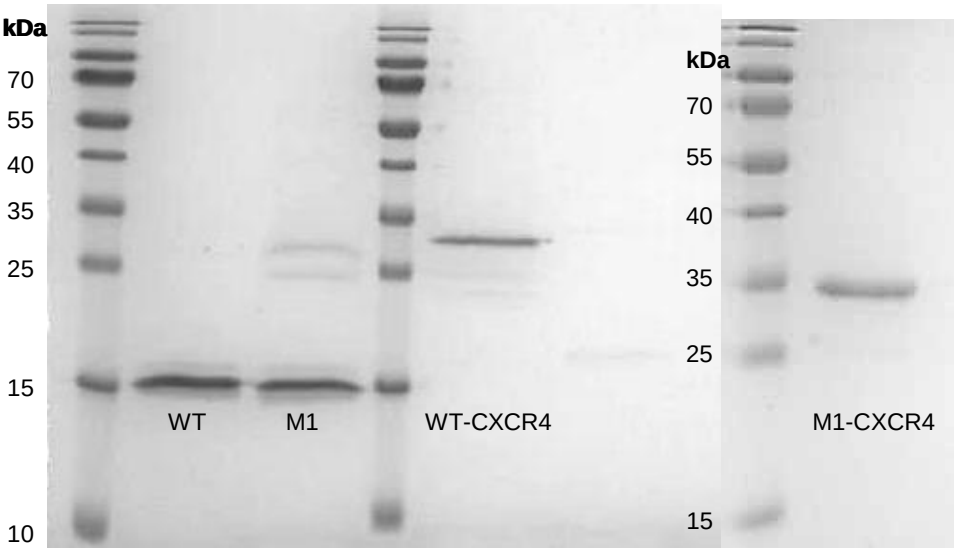

Figure 8 - SDS-PAGE analysis of purified VHH monomers and heterodimers proteins. Gel was stained with Coomassie brilliant blue.

Purification yields for the different proteins are shown on table 2:

Table 2 – Yield of purified recombinant proteins for 500mL of bacterial culture.

Protein	Total protein amount (µg)
WT	240
M1	192
WT-CXCR4	2310
M1-CXCR4	390

3.2. Binding of the VHH heterodimers to methotrexate

After purification it was necessary to verify if the constructs continue to recognize methotrexate. Preliminary binding assays were performed by ELISA (see section 5. from materials and methods) using bovine serum albumin-methotrexate conjugate (BSA-MTX) as antigen.

Results shown in figure 9 demonstrate that anti-MTX WT monomer and anti-MTX WT-CXCR4 heterodimer specifically bind to methotrexate in a concentration-dependent manner while no binding was detected to BSA, even for the highest concentrations of protein. However, anti-MTX M1 and anti-MTX M1-CXCR4 shows higher cross-reaction to BSA alone when comparing to anti-MTX WT and anti-MTX WT-CXCR4 which bind selectively to methotrexate. Moreover, anti-MTX M1-CXCR4 is less specific than the monomer anti-MTX M1.

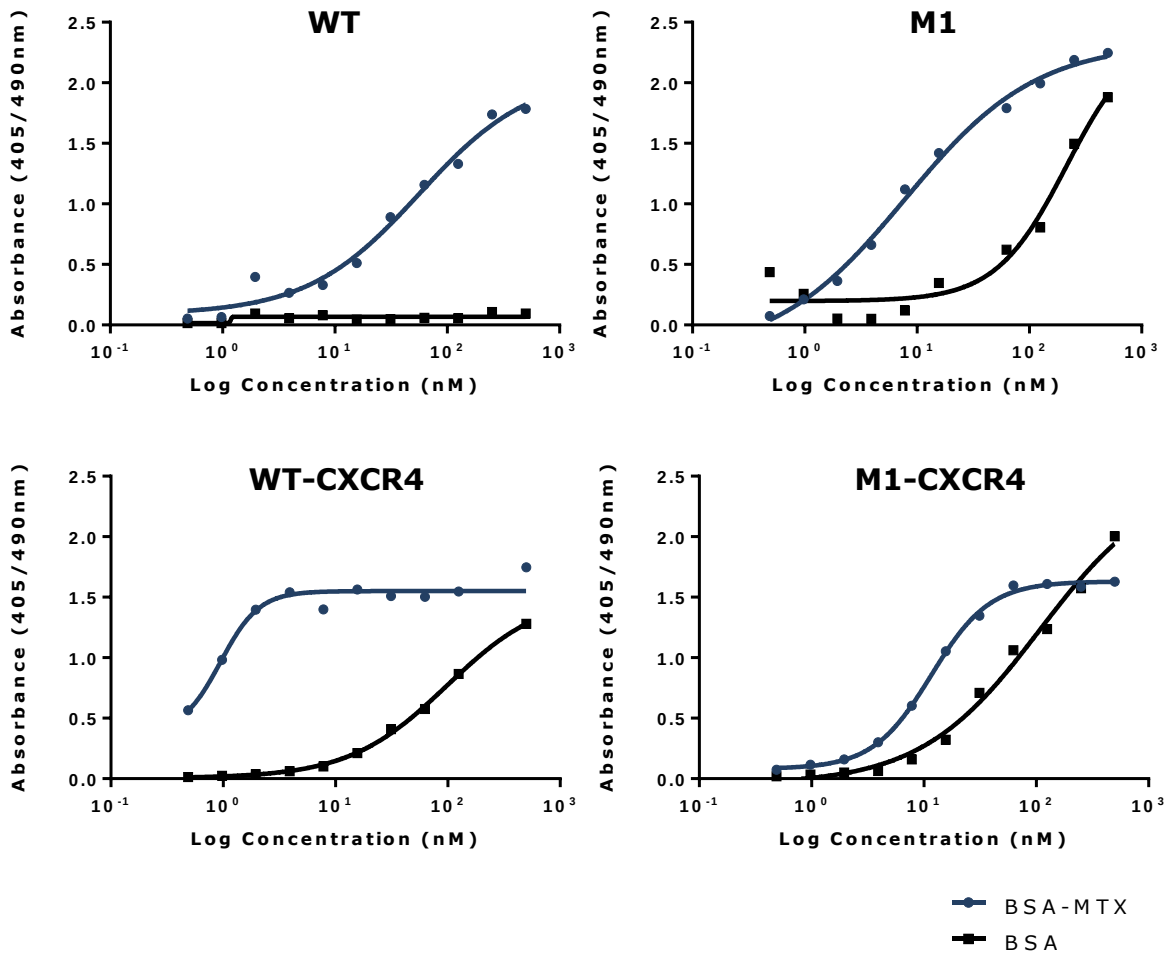

Figure 9 - ELISA assays to evaluate binding of VHH monomers and heterodimers to methotrexate. Assay was performed using 2 µg per well of BSA-MTX as antigen. 3 % BSA (PBS) was used for blocking and as a negative control. Serial dilutions of purified proteins were diluted in 1 % BSA (PBS). Detection was achieved using an HRP-conjugated HA-tag or FLAG-tag antibodies according to each sample.

3.3. Determination of antibody specific cleavage by metalloproteases

After the verification that recombinant proteins are specific to MTX, another functional assay was performed to determine whether MMP-9 can cleave the antibody and release MTX.

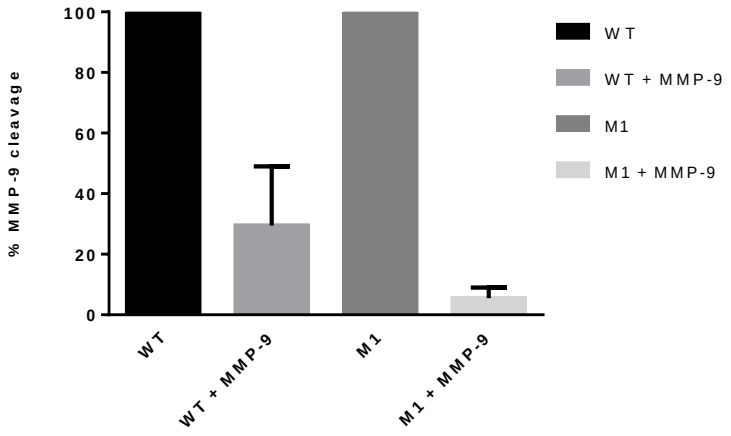

Figure 10 – Determination of antibody specific cleavage by MMP-9. WT and M1 were incubated with MTX-FITC for 1h at 37ºC. Afterwards, conjugates were exposed to nickel-charged resin for 1h. MMP-9 was added to the previous mixture and fluorescent intensity was measured. Values were normalized to WT and M1 without MMP-9.

The results reveal that fluorescent intensity decrease in the presence of MMP-9 in both VHH nanobodies (anti-MTX WT and M1). However, values are even lower in anti-MTX M1 since it has a MMP-9 cleavage site in the antibody sequence (figure 10).

3.4. Binding of VHH heterodimers to CXCR4-expressing cells

Since VHH heterodimers are bispecific proteins, binding of each functional domain to its target must be assessed. Therefore, after evaluation of specific binding of the VHH heterodimers to methotrexate, binding to CXCR4 was evaluated by flow cytometry using CXCR4-expressing cell lines.

As previous mentioned, CXCR4 receptor is internalized and re-expressed at the cell surface after the ligand binding. Due to this behavior, the assays were performed at 4 ºC and 37 ºC, to evaluate surface binding and internalization, respectively.

As the binding and internalization assays were performed with MTX-FITC, the target cells changing its fluorescence emission spectrum to green (525 nm) and it allows to be detected by flow cytometry.

Regarding CXCR4-surface binding, anti-MTX WT-CXCR4 bind specifically to CXCR4 receptor (51 % of FITC positive cells). Anti-MTX WT-CXCR4 presented a higher binding ability than anti-MTX M1-CXCR4 and also higher than the controls (anti-MTX WT and M1), which was expected since controls did not have the anti-CXCR4 VHH domain. The actual number of FITC positive cells was 16.5 % for anti-MTX M1-CXCR4, 9.4 % for anti-MTX WT and 14.8 % for anti-MTX M1 (figure 10).

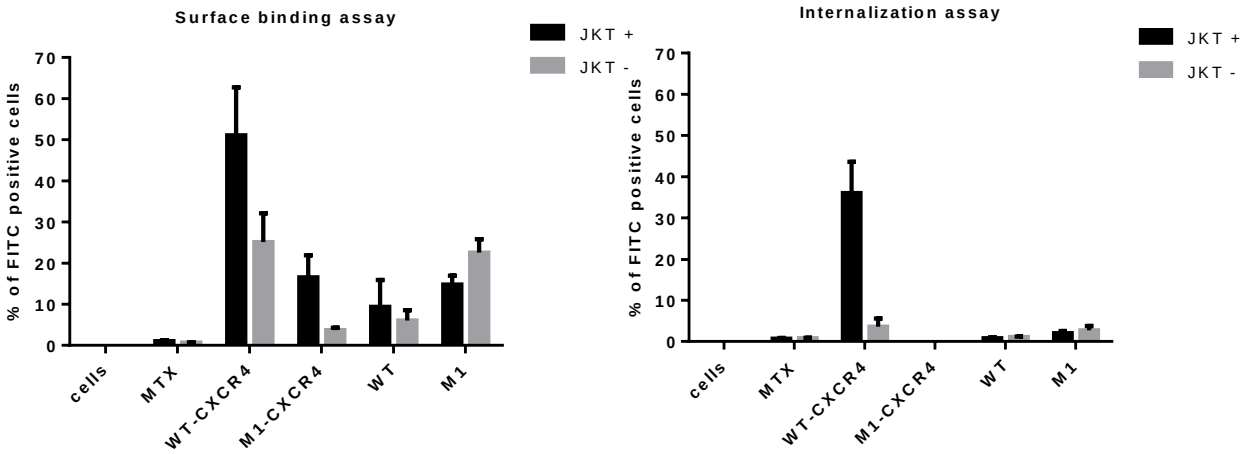

Figure 11 - Graphical representation of flow cytometry assay to evaluate VHH heterodimers binding of CXCR4 receptor. Values represent mean ± SEM of three independent assays.

For CXCR4 internalization assay, anti-MTX WT-CXCR4 is the only protein which can internalize via CXCR4 receptor (3.6 % of FITC positive cells). There are no results available for the internalization of anti-MTX M1-CXCR4 because this protein compromised cell viability. Regarding controls, despite anti-MTX WT/M1 presented low binding ability at the surface (around 10 % of FITC positive cells) the results for internalization are even lower (0.8 % for anti-MTX WT and 2 % for anti-MTX M1) (figure 10).

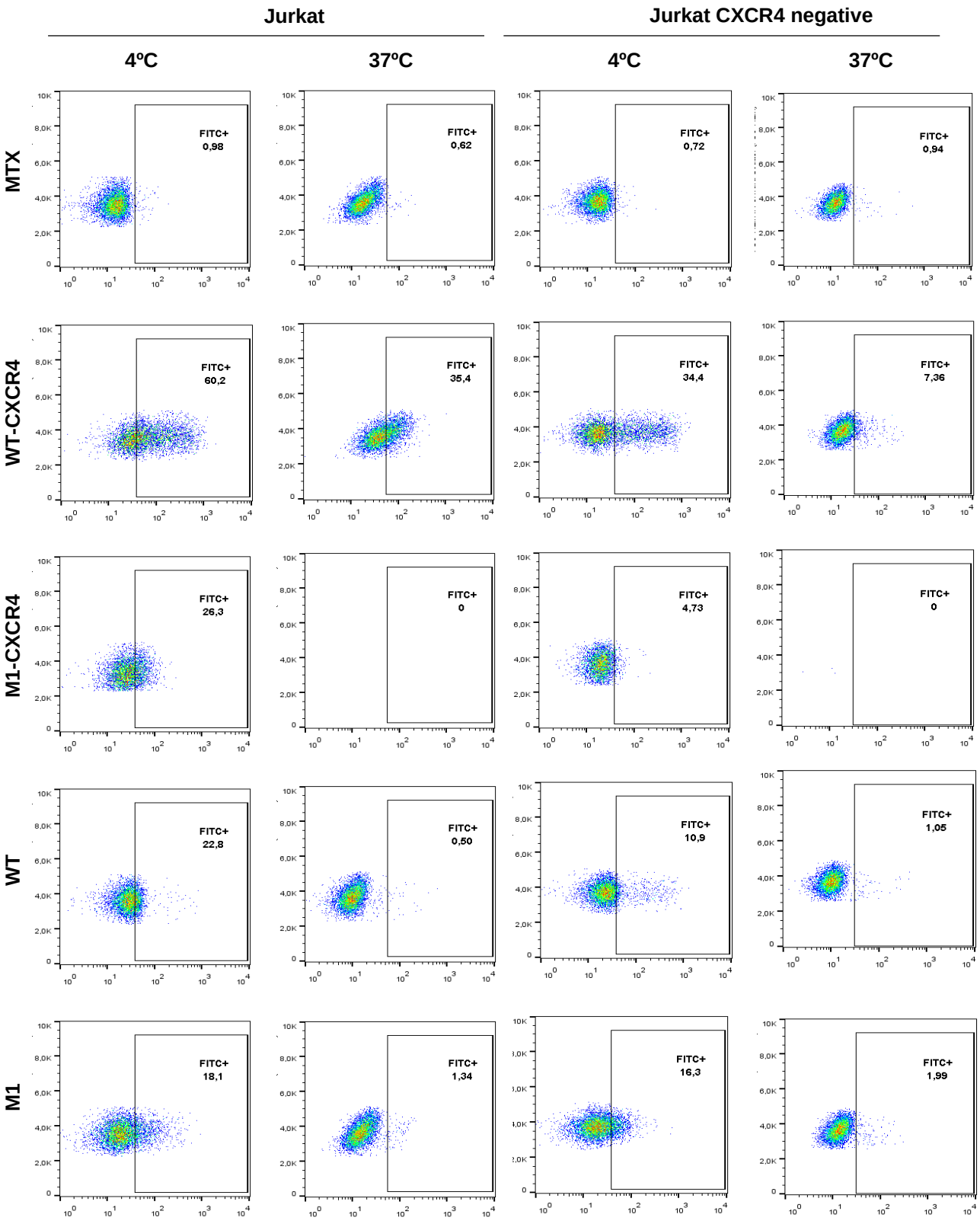

Figure 12 - Flow cytometry assay to evaluate VHH heterodimers binding to CXCR4.
For each assay condition, Jurkat E6- 1 were seeded at 2x10^5 per well in 96-well plates. Recombinant proteins (2µM) were incubated with methotrexate-fluorescein isothiocyanate conjugate (MTX-FITC) (1µM) at 37°C for 1 h. Afterwards, the proteins plus MTX-FITC were incubated at 4°C or 37°C for 2 h. Following 2 h of incubation, cells which were incubated at 37°C were washed twice with trypsin and then with PBS. Cells which were incubated at 4°C were washed twice with PBS. MTX-FITC and anti-MTX VHH (WT and M1) were used as controls. Bispecific VHH heterodimers binding to CXCR4 were detected by excitation at 488 nm and detection at 525 nm. Flow cytometry analysis was performed in Guava® easyCyte HT, by acquirement of 5000-gated events from each sample. Values are relative to Jurkat cells treated with MTX-FITC. This figure is representative of three assays performed.

To confirm that internalization of VHH heterodimers is via CXCR4 receptor, the same assay was done in a Jurkat CXCR4 negative cell line (see section 2.9. from materials and methods).

Anti-MTX WT-CXCR4 and anti-MTX M1 presented unspecific surface binding (25.1 % and 22.6 %, respectively) in a Jurkat CXCR4 negative cell line. However, concerning to internalization results for the same cell line, anti-MTX WT-CXCR4 is the only protein that internalize via CXCR4 (36%). The other three recombinant proteins showed low percentages of FITC-positive cells (1.1 % for anti-MTX WT and 2.7 % for anti-MTX M1) (figure 10). Anti-MTX M1-CXCR4, as well as in Jurkat CXCR4 positive cell line, continues to induce cell death.

According to the results obtained in Jurkat CXCR4 positive and negative cell lines, it was shown that anti-MTX WT-CXCR4 could specifically bind and internalize via CXCR4 receptor which is validated by internalization assay in Jurkat CXCR4 negative cell line and by negative controls. In addition, anti-MTX M1-CXCR4 induce cell death in both Jurkat cell lines by a mechanism which is not completely understood.

3.5. Anti-MTX M1-CXCR4-induced cell death

To understand by which mechanism anti-MTX M1-CXCR4 can induce cell death, different types of assays were performed. Since anti-MTX M1-CXCR4 has a mutant 1 anti-MTX VHH domain which has a MMP-9 cleavage site, cell death upon MTX release was evaluated.

First, a flow cytometry assay was performed in which only anti-MTX M1-CXCR4 (with and without previously MTX conjugation) was incubated with Jurkat cells. The recombinant protein induced cell death in these conditions (figure 13), suggesting a mechanism independent of MTX presence and release.

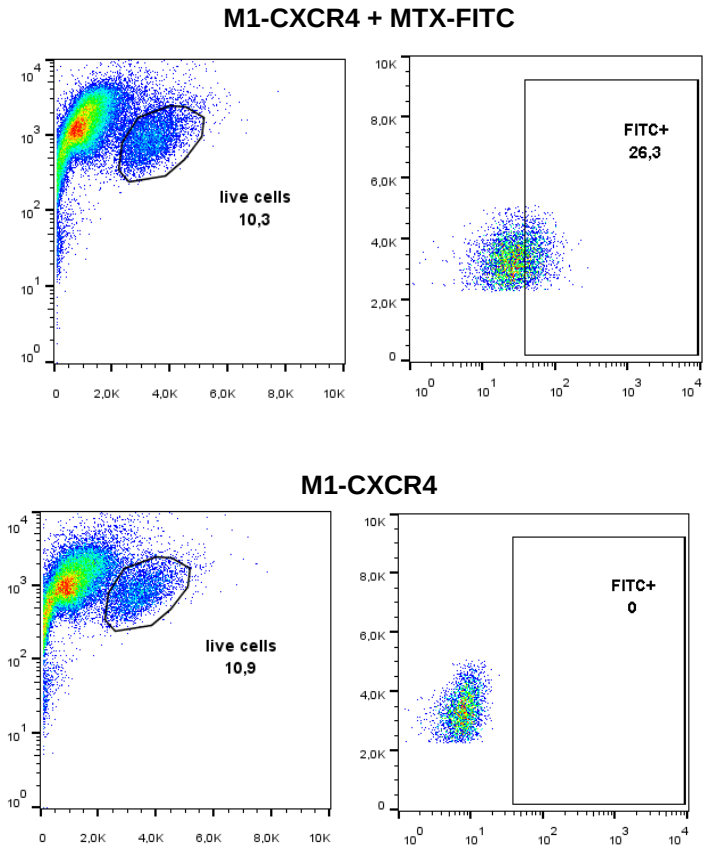

Figure 13 - Flow cytometry assay to evaluate if anti-MTX M1-CXCR4 toxicity is MTX-dependent. For each assay condition, Jurkat E6- 1 were seeded at $2x10^5$ per well in 96-well plates. Anti-MTX M1-CXCR4 (2 µM) were incubated with and without methotrexate-fluorescein isothiocyanate conjugate (MTX-FITC) (1 µM) at 37 °C for 1 h. Afterwards, the proteins were incubated at 4 °C for 2 h. Following 2 h of incubation, cells were washed twice with PBS. Bispecific VHH heterodimers binding to CXCR4 were detected by excitation at 488 nm and detection at 525 nm. Flow cytometry analysis was performed in Guava® easyCyte HT, by acquirement of 5000-gated events from each sample. Values are relative to Jurkat cells treated with MTX-FITC.

Secondly, serial dilutions of anti-MTX M1-CXCR4 and WT-CXCR4 (used as a control) were performed in Jurkat cell line (see section 2.9. from materials and methods) and analyze by flow cytometry (figure 14). Anti-MTX M1-CXCR4 induced cell toxicity in a concentration-dependent manner. However, at 1 µM (half of concentration used in CXCR4 binding and internalization assays) the percentage of live cells increased (70 %) but the percentage of FITC positive cells decreased (0.27 %), suggesting that the protein can no longer bind to CXCR4 at that concentration.

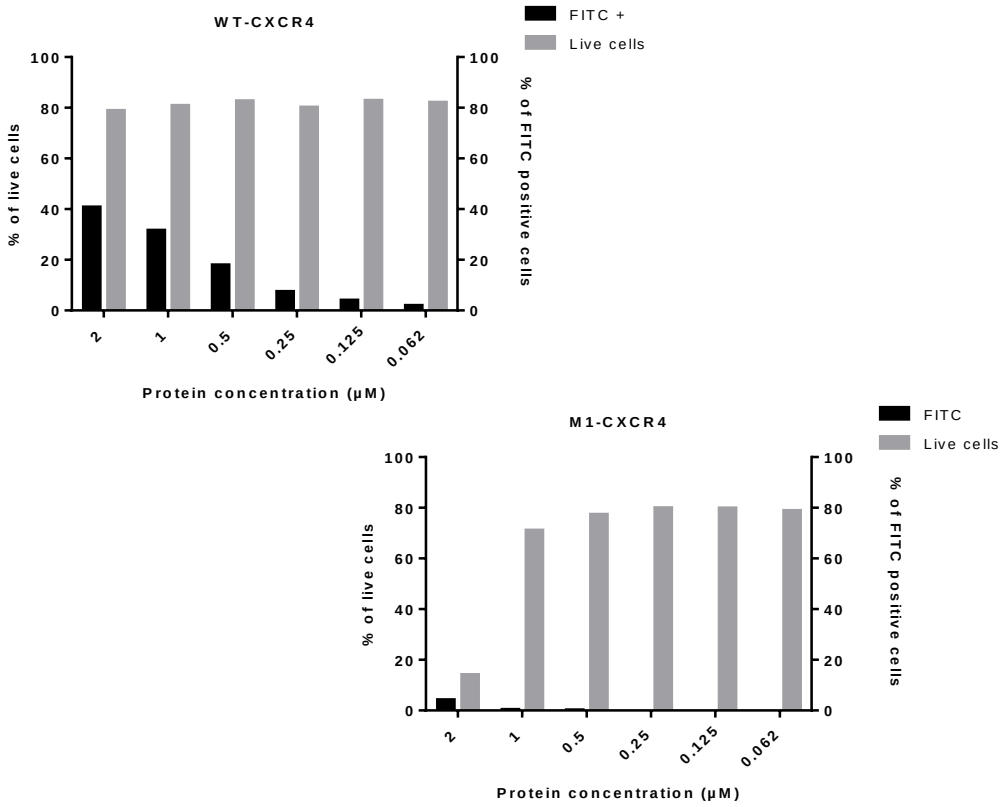

Figure 14 - Flow cytometry assay to evaluate anti-MTX M1-CXCR4 toxicity. For each assay condition, Jurkat E6- 1 were seeded at 2x105 per well in 96-well plates. Anti-MTX M1-CXCR4 and WT-CXCR4 (2µM) were incubated with methotrexate-fluorescein isothiocyanate conjugate (MTX-FITC) (1µM) at 37° C for 1 h. Afterwards, the proteins were incubated at 4° C for 2 h. Following 2 h of incubation, cells were washed twice with PBS. Bispecific VHH heterodimers binding to CXCR4 were detected by excitation at 488 nm and detection at 525 nm. Flow cytometry analysis was performed in Guava® easyCyte HT, by acquirement of 5000-gated events from each sample. Values are relative to Jurkat cells treated with MTX-FITC.

To understand which type of cell death (apoptosis or necrosis) was induced by anti-MTX M1-CXCR4, a lactate dehydrogenase (LDH) and caspases activation assay were kindly performed by Marta Afonso (Cecília Rodrigues group). The results, in particular the LDH release, suggest that cells are dying by mechanisms inductors of necrosis (figure 14).

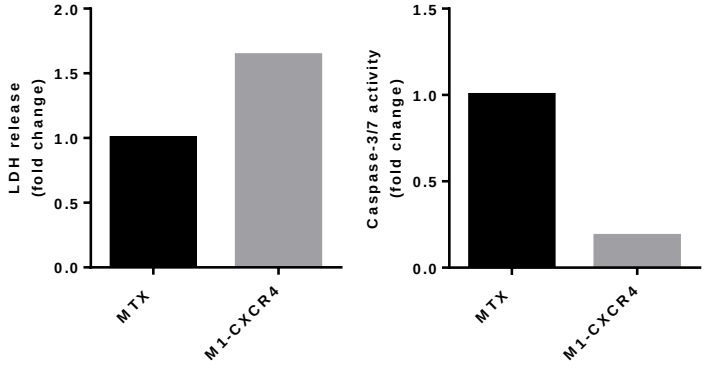

Figure 15 - LDH and caspase-3/7 activation assays to evaluate cell death induced by anti-MTX M1-CXCR4. Results are expressed as fold change of MTX treated cells.

3.6. Assessment of cell viability in the presence of VHH heterodimers

After the initial characterization of our constructs, the *in vitro* efficacy of the antibody drug conjugates was evaluated in Jurkat cells by MTT assay for 24h, 48 h and 72h.

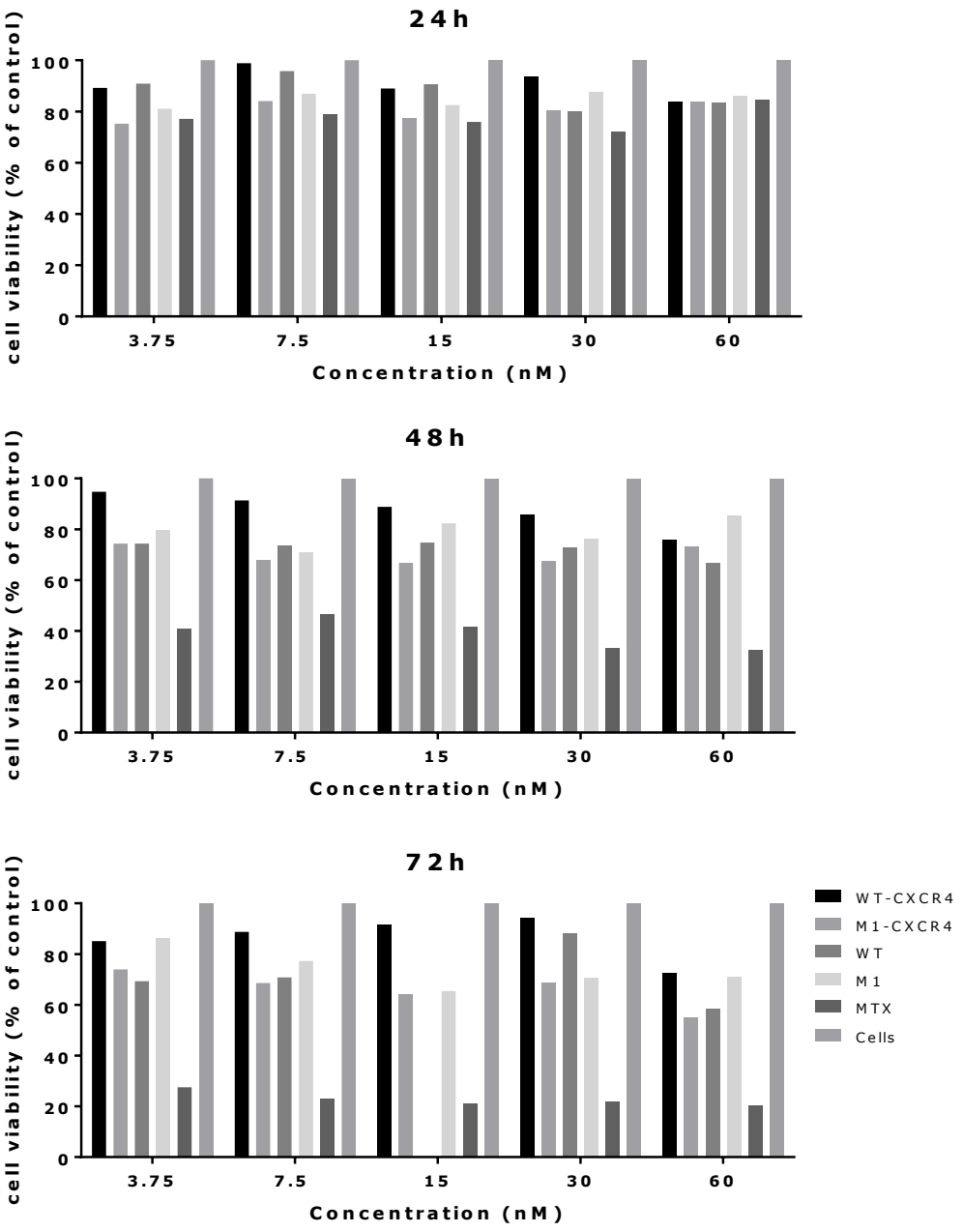

Figure 16 - In vitro cytotoxicity assay to evaluate the efficacy of the bispecific VHH heterodimers. Jurkat E6-1 were seeded at $2x10^5$ per well in 24-well plates. After 1h incubation of recombinant proteins with MTX, repeated washes with PBS were performed in order to purify the antibody drug conjugates. MTT assay was performed at three time points (24h, 48h and 72h). The absorbance of dissolved formazan crystals was measured at 590 nm using a microplate reader. Since the absorbance directly indicates the number of viable cells, percent viability was calculated directly from the absorbance values.

The results shown in figure 14 suggest a decrease in the percentage of live cells treated with recombinant proteins. In addition, at 72 h and at the highest concentration of antibody-drug conjugate, anti-MTX M1-CXCR4 leads to 45 % of cell death while anti-MTX WT-CXCR4 (28 %), anti-MTX WT (40 %) and anti-MTX M1 (30 %) showed lower percentages.

3.7. Construction and expression of Trastuzumab plus anti-MTX WT/M1

For the development of an additional therapeutic strategy for antibody drug delivery, we constructed a bispecific protein which contains a monoclonal antibody that interferes with the HER-2 receptor (Trastuzumab, Herceptin ®) fused to an anti-MTX VHH (anti-MTX WT and anti-MTX M1 described in section 2.11. from materials and methods).

After the transfection of HEK293T cells, supernatants were recovered and immunoprecipitation was performed with native Protein A sepharose. To confirm protein expression in this cell line, a Western Blot assay was conducted with the total protein of each sample.

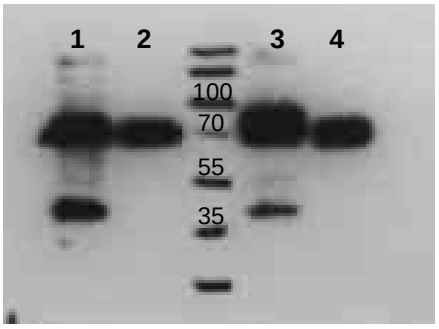

Figure 17 - Western Blot analysis of transfection of Trastuzumab plus anti-MTX WT/M1 in HEK293T cell line. **Legend:** 1. Supernatant Trastuzumab+WT; 2. Lysate Trastuzumab+WT; 3. Supernatant Trastuzumab+M1; 4. Lysate Trastuzumab+M1

The fusion protein obtained show the expected molecular weight of ~150 kDa. Although supernatants and lysates from transfection cells showed detectable amounts of both proteins, the protein expression level is higher in the supernatant fractions.

DISCUSSION

Monoclonal antibodies represented a consolidated therapeutic approach over the past two decades. To improve their potency and selectivity, there is a vast interest in arming antibodies with potent cytotoxic drugs in the form of antibody drug conjugates (ADCs).

Significant progress has been made in developing ADCs for the selective delivery of cytotoxic drugs to tumors reducing non-specific side effects. Additionally, important advances have been made to engineer antibody fragments. These antibody fragments exhibit several advantages in comparison with conventional antibodies, making them promising tools for diagnosis and therapy.

The purpose of this thesis is the development of a new strategy for antibody drug delivery targeting the tumor microenvironment. For this, we engineered and constructed a bispecific VHH heterodimer composed by an anti-methotrexate VHH and an anti-CXCR4 VHH. To validate our strategy, three anti-MTX VHH were synthetized as monomers. Anti-MTX VHH WT was selected by panning from an immune-llama library using phase display technology by Alvarez-Rueda [69]. The other two nanobodies also included a sequence encoding a MMP-9 cleavage site in two different positions in the VHH framework. Since MMP-9 is an extracellular proteinase which is overexpressed in several tumor types, it was included in the constructs in order to facilitate the release of methotrexate in the target cells.

Initially, a fragment encoding anti-MTX VHH WT, M1 and M2 were amplified by PCR and subcloned into the bacterial expression vector pET-21a(+). After that, the bispecific VHH heterodimers were constructed. For this, an anti-CXCR4 VHH was linked to an anti-MTX VHH. The CXCR4-specific nanobody was generated by phage display by Sven Jähnichen et al. [70].

The two possible constructs for each anti-MTX VHH were designed in order to determine which one shows better solubility and stability. One of them was constructed with the anti-MTX on the N-terminal and the other one on the C-terminal. All constructs were subcloned into the bacterial expression vector pET-21a(+). All the recombinant proteins were transformed in *E.Coli* bacterial strains. Regarding the monomers, it was necessary to optimize the conditions for recombinant protein expression. For this, small scale expression and test purification was performed. The soluble and insoluble protein fractions were applied to SDS-PAGE. Anti-MTX WT presented high protein expression in the soluble fraction in contrast to anti-MTX M1 which presented a single band in the insoluble fraction.

The reason why recombinant proteins are recovered from the insoluble fraction is because they are expressed in inclusion bodies [75].

Anti-MTX M2 presented a residual protein expression in both fractions. This is an indicator that not all positions on VHH framework can support MMP-9 cleavage sites, probably because it plays an important role in antibody stability and solubility. For this reason, the future assays were conducted only with anti-MTX WT and M1.

The same optimized procedures were performed with the bispecific VHH heterodimers (anti-MTX WT-CXCR4, anti-MTX M1-CXCR4, anti-CXCR4-MTX WT, anti-CXCR4-MTX M1). Once separated by electrophoresis, the results showed that the constructs in which the anti-MTX was at the N-terminus produced a high amount of protein. For this reason, the future assays were conducted only with these constructs (anti-MTX WT-CXCR4 and anti-MTX M1-CXCR4). In concordance with the results obtained for the monomers, the anti-MTX WT-CXCR4 showed a higher protein expression in the soluble fraction in contrast to anti-MTX M1-CXCR4 which presented a single band in the insoluble fraction.

Although SDS-PAGE results showed a single protein band with the expected molecular weight for both recombinant proteins (~35 kDa), anti-MTX WT-CXCR4 presented a low yield compared with anti-MTX M1-CXCR4. To overcome this problem, anti-MTX WT-CXCR4 was transformed in Shuffle *E.Coli* strain which is engineered to form proteins containing disulfide bonds [76]. With this *E.Coli* strain it was possible to increase the amount of protein produced.

After the optimization of the expression conditions for all the constructs it was necessary to purify the recombinant proteins. Since a histidine tag (His8) was included in the constructs on the C-terminal it was possible to purify by Immobilized Metal Affinity Chromatography (IMAC) with a nickel column. Afterwards, the eluted proteins were submitted to a buffer exchange to a more suitable storage buffer. SDS-PAGE and Western Blot results showed a single protein band with the expected molecular weights for the recombinant proteins under reducing conditions and protein concentration was determined by Bradford method.

The total amount of purified proteins was 240 µg for anti-MTX WT, 192 µg for anti-MTX M1, 2310 µg for anti-MTX WT-CXCR4 and 315 µg for anti-M1-CXCR4.

These results show that the initial aim of this thesis was successfully conducted.

After expression and purification of recombinant proteins it was necessary to evaluate if the protein function was maintained. For this, it was necessary to verify if the

constructs could bind to methotrexate. Since methotrexate is a small molecule and for this reason is not possible to attach it onto a plate, a strategy using immobilized methotrexate was followed. For the preliminary binding assays which were performed by ELISA, bovine serum albumin-methotrexate conjugate (BSA-MTX) was prepared via carbodiimide linkage and used as antigen [69]. Results revealed that anti-MTX WT-CXCR4 could specifically bind to methotrexate. However, recombinant proteins in which anti-MTX M1 was present showed an increasing of nonspecific interactions with BSA alone. One of the reasons which explained these results the insertion of a MMP-9 cleavage site in the anti-MTX M1 framework, which influences protein conformation. The conformational changes may change the antigen-binding site of the nanobody. In addition, fusion of anti-MTX VHH monomer into a heterodimeric construct (in this case, with an anti-CXCR4 VHH) contributes, by itself, to nonspecific interactions against BSA alone.

After the evaluation of specific binding to methotrexate by ELISA assay, another preliminary functional assay was performed in order to verify if MMP-9 can cleave the antibody and release methotrexate. Since anti-MTX WT and anti-MTX M1 are conjugated to MTX-FITC, fluorescent intensity was expected to decrease upon methotrexate release from the antibody. Our results demonstrated that fluorescent intensity decrease in the presence of MMP-9 in both VHH nanobodies (anti-MTX WT and M1). However, and as expected, values are even lower in the anti-MTX M1 since it has a MMP-9 cleavage site included in the VHH framework. Since VHH heterodimers are bispecific proteins it was necessary to evaluate binding properties of both functional domains.

CXCR4 is a transmembrane receptor and because of this all the assays that involves CXCR4 were performed in cells. Since CXCR4 is internalized and re-expressed at the cell surface, all assays were performed at 4 ºC (for cell surface binding) and 37 ºC (for internalization) [63]. In addition, we choose Jurkat cell line as model cells since they constitutively express CXCR4 receptor [77]. Regarding anti-MTX WT-CXCR4, it could bind specifically to CXCR4 at the surface and internalize via this receptor. In addition, in a Jurkat CXCR4 negative cell line, although there is an unspecific surface binding there is no receptor internalization.

These findings indicate that with this protein is possible to target CXCR4 specifically and all antibody-drug conjugate could internalize via this receptor.

During the time of assay, the methotrexate did not have time to induce cell death since the assays with this cytotoxic drug are performed during 48 h – 72 h [78]. This is the

reason why it is not possible to evaluate the cell death and only functionality with this type of assay. In relation to anti-MTX M1-CXCR4, there are no results available because the protein induces cell death. Although, in the CXCR4 surface binding assay, it was possible to detect FITC positive cells but it was not statistically significant since only 5% of cell population is alive. In addition, in a Jurkat CXCR4 negative cell line, the same result was obtained. These results showed that, regardless the presence or absence of CXCR4 receptor, there is a mechanism by which this protein induces cell death.

Since nanobodies by itself present *in vitro* and *in vivo* low toxicity profiles and there are other studies in which this antibody fragment was used as a therapeutic agent it is important to understand by which mechanism anti-MTX M1-CXCR4 induces cell death [17,79]. To achieve this, several types of assays were performed. Since anti-MTX M1-CXCR4 has a MMP-9 cleavage site in the anti-MTX M1 monomer, it was necessary to verify if the cell death is related to MTX release into the target cells.

First, a flow cytometry assay in which anti-MTX M1-CXCR4 (without previously incubated with MTX) was incubated with cells. Results reveal that the protein continues to induce cell death and is independent of MTX presence and release.

Secondly, serial dilutions of anti-MTX M1-CXCR4 and anti-MTX WT-CXCR4 were performed. Results showed that cell toxicity is induced in a concentration-dependent manner. However, when we used half of the concentration in CXCR4 binding and internalization assays the percentage of live cells was increased but the protein can no longer bind to CXCR4 which indicate that the decrease in toxicity was accompanied by a loss of functionality. Finally, to conclude which type of cell death (apoptosis or necrosis) was induced by anti-MTX M1-CXCR4, a LDH and caspases activation assay were performed. The results reveal that these cells suffer necrotic death.

In conclusion, although it is necessary to perform additional assays to understand by which mechanism anti-MTX M1-CXCR4 induces cell death, these findings demonstrate that this protein induces necrosis, which means that is toxic by itself and it is not dependent of MTX coupling. In addition, the cell toxicity is in a concentration-dependent manner.

Taking into account all the promising results based on this antibody drug delivery system, a preliminary *in vitro* cytotoxicity assay was performed in Jurkat cell line. Jurkat cells in PBS were taken as control with a cell viability of 100%. Cells were incubated with the different recombinant proteins and with free MTX as a positive control for 72 h. Results showed that, up to 72 h of incubation, is evident a decrease in the percentage of cell viability when treated with recombinant proteins. In addition, and as expected,

cells treated with free MTX presented an even greater decrease in cell viability. At the highest antibody-drug concentration, anti-MTX M1-CXCR4 leads to 45% of cell death while anti-MTX WT-CXCR4 (28%), anti-MTX WT (40%) and anti-MTX M1 (30%) showed lower percentages. Regarding free MTX, only 20% of cells were alive. These findings indicate that although free MTX continues to efficiently kill cells, this antibody-drug conjugate strategy has also an effective activity to induce cell death.

Simultaneously, and due to fact that antibody fragments (e.g. nanobodies) exhibit several limitations in pharmacokinetics, such as a rapid clearance and large volume of distribution, other therapeutic strategy was development. We designed and constructed bispecific proteins, which contain a monoclonal antibody that interferes with the HER-2 receptor (Trastuzumab, Herceptin ®) fused to an anti-MTX VHH (anti-MTX WT and M1). Preliminary transfections assays and a western blot showed that recombinant proteins were successfully constructed and expressed in HEK293T cell line.

In conclusion, the strategy for antibody-drug delivery developed in this study is a promising system to delivery cytotoxic drugs using the tumor microenvironment specific properties to enhance their efficiency.

Conclusions and Future Perspectives

Antibody-drug conjugates are an emerging class of biopharmaceuticals that combines the specificity of monoclonal antibodies to deliver cytotoxic drugs selectively to a target antigen.

Monoclonal antibodies have demonstrated to have an essential role in cancer treatment becoming the standard of care in several types of tumors. In addition, classic chemotherapy demonstrates reduced selectivity against cancer cells leading to a small therapeutic window. ADCs combine these two two classes of drugs leading to a highly selective and highly cytotoxic cancer treatment. Although ADCs have been under investigation for decades, only two ADCs have been approved by FDA and EMA.

Understanding how to enhance each ADCs components contributes to the efficacy and safety of this new therapeutic approach. Targeting the tumor microenvironment and include small antibody fragments are promising strategies for the development of future ADCs.

To overcome all the limitations presented in the previous ADCs, in the present work we develop a new strategy for antibody drug delivery. For this, we constructed a bispecific VHH heterodimer against an overexpressed tumor antigen and a cytotoxic drug. To enhance the specificity to the tumor microenvironment, in one of the constructs it was also include a sequence encoding a MMP-9 cleavage site to facilitate the release of cytotoxic drug in the target cells.

The development of this new ADC strategy is divided in 3 parts. The first part was the construction of the bispecific VHH heterodimers which is composed by an anti-MTX VHH and an anti-CXCR4 VHH. The monomers of anti-MTX VHH, with and without a MMP-9 cleavage site, were also constructed and used as controls.

All the constructions were successfully engineered and optimized for bacterial expression and IMAC purification with high yields of purified soluble protein.

In the second part, we performed *in vitro* assays to characterize VHH monomers and heterodimers in relation to their specificity and affinity. For this, preliminary binding assays were performed by ELISA using BSA-MTX as antigen. We demonstrated that both monomers and one of the VHH heterodimers (anti-MTX WT-CXCR4) bind specifically to MTX in a concentration-dependent manner. In addition, other functional assay showed that the presence of MMP-9 cleavage site induce antibody cleavage and MTX release.

Since VHH heterodimers are bispecific proteins it was always necessary to evaluate both functional domains. For this reason, a flow cytometry assay was performed in Jurkat E6-1 T cell line which overexpressed CXCR4 receptor. Regarding anti-MTX WT-

CXCR4, it is possible to verify that this protein could specifically bind at the surface and internalize through CXCR4 receptor. Regarding anti-MTX M1-CXCR4, this protein induces cell death by a mechanism which is not completely understood. Preliminary assays showed that cell death in not dependent of MTX presence and release and it toxicity is in a concentration-dependent manner in which a decrease in toxicity result in loss of function. In addition, cell death assays demonstrated that cells suffer necrosis.

In the third part, *in vitro* cell cytotoxic assay to evaluate ADC function. MTT assay were performed with recombinant proteins at different concentrations and free MTX as a positive control. Results showed an evident decrease in the percentage of viability of cells for all the constructs with anti-MTX M1-CXCR4 presented an even greater decrease in cell viability.

Regarding Trastuzumab + WT/M1, preliminary assays demonstrated that these recombinant proteins were successfully constructed and expressed in HEK293T cell line.

In conclusion, the antibody drug conjugates developed in this thesis are an innovative and promising therapeutic strategy that takes advantage of the specific properties of the tumor microenvironment to delivery cytotoxic drugs to cancer cells.

As a future perspective, it will be necessary to develop other assays in order to understand the mechanism by which anti-MTX M1-CXCR4 can induce cell death by itself. In addition, we will perform at least three independent assays for assessment of cell viability in the presence of bispecific VHH heterodimers (MTT assay) having a higher protein concentration and a more specific method for MTX conjugation and purification. For the binding and internalization assays, it will be necessary to construct a VHH heterodimer which includes a VHH irrelevant to prove, in other way, that the binding and internalization is CXCR4 receptor depending

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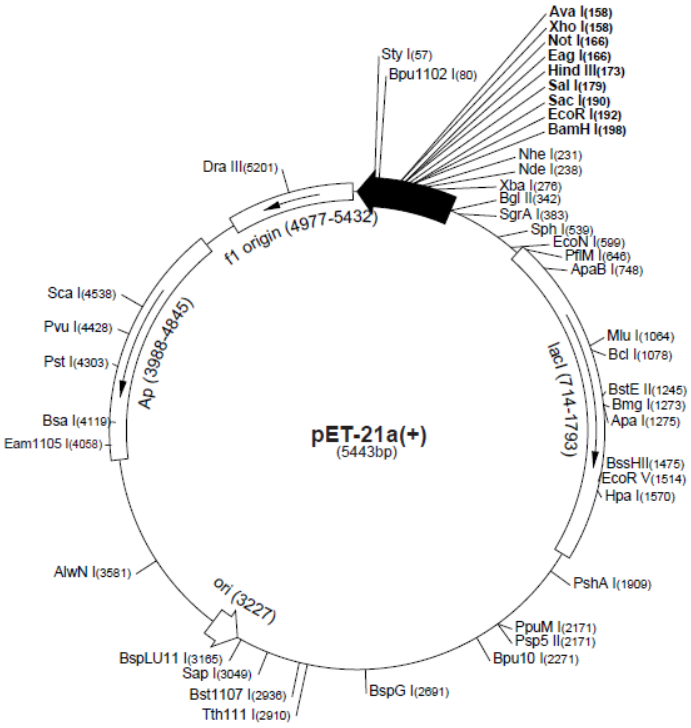
ANNEXES

Table 3 – Primers sequence used in PCR reactions.

Primer name	Sequence 5'-3'
Primer 1: VHH-CXCR4-R-XhoI	CCGCTCGAGTCATGCGTAATCAGGCACGTCGTAGG GGTACGATCCATGGTGATGGTGATGGTGATGGTGG CTGCCTCCGCCTCCACTGCTGCTCACGGTCACCTG
Primer 2: VHH anti-CXCR4-linker-SACI R	CGAGCTCGCTTCCGCCTCCTCCGCTGCTCACGGTC ACCTG
Primer 3:VHH anti-CXCR4-NheI-F	CTAGCTAGCGCGGTGCAGCTGGTGGAG
Primer 4: VHH-MTX-SACI-F	CGAGCTCCAGGTGCAGCTGGTGCAG
Primer 5: VHH-MTX-Nhe-F	CTAGCTAGCCAGGTGCAGCTGGTGCAG
Primer 6: VHH-MTX-Fusion-Fc-R	ACCCGGAGACAAGCTAGTCACTTGTCGTCATCGTC TTTG
Primer 7: VHH-MTX-Fusion-Fc-F	AGAAATCACTAAGCTTGGCTAGCCAGGTGCAGC
Primer 8: MTX-CXCR4-Nhe-R	GCTGCACCTCGCTAGCGCTGCCTCCGCCTCC
Primer 9: MTX-CXCR4-Nhe-F	TATACATATGGCTAGCCAGGTGCAGCTG

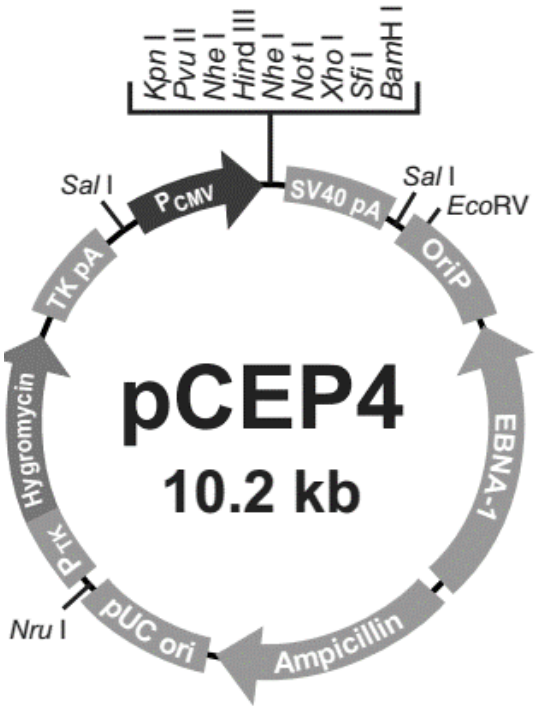
Table 4 – PCR conditions program used in PCR reactions

Phusion Green High-Fidelity DNA Polymerase			
Cycle Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98ºC	30 s	1
Denaturation	98ºC	10 s	30
Annealing	60ºC	30 s	
Extension	72ºC	30 s	
Final extension	72ºC	10 min	1



Plasmid Features
T7 promoter
T7 transcription start
T7-tag coding sequence
Multiple cloning sites
His-tag coding sequence
T7 terminator
lacI coding sequence
pBR322 origin
bla coding sequence
f1 origin

Figure 18 - Plasmid features and genomic map of pET-21a(+).



Plasmid Features
CMV promoter
Multiple cloning site
SV40 polyadenylation signal
Ampicillin resistance gene
pUC origin
Hygromycin resistance gene
OriP
EBNA-1 gene
TK promoter
TK polyadenylation signal

Figure 19 - Plasmid features and genomic map of pCEP4.