ANTIBODY-DRUG CONJUGATES (ADCS), THE NEW ANTITUMOR THERAPY. ANALYTICAL METHODOLOGIES FOR THEIR DETERMINATION

Lidia Blasco[1,2], Eider Alcaraz[2], Nicholas King[2] Rosa M. Alonso[1], Nerea Leal[2]

[1] Departamento de Química Analítica, Facultad de Ciencia y Tecnología, Universidad del País Vasco (UPV/EHU), Barrio de Sarriena, s/n, 48940, Leioa, Bizkaia; [2] CTI Laboratory Services, Parque Tecnológico de Bizkaia, Ed.801 B, 1ª Planta, 48160 Derio, BizKaia.

The cancer is one of the leading causes of death in the world, producing 10 million of deaths in 2020. Although many of these deaths could not have been avoided, there are some that could, with an early diagnosis and advanced treatments (1). The chemotherapy was developed as a strategy against cancer during the 1940s by Goodman´s team and it brought great hope for a cure against cancer, but without forgetting the high levels of systemic toxicity. In the 1970s, the use of monoclonal antibodies as an alternative treatment started to develop thanks to Milstein and Köhler, which gave rise to a decrease in systemic side effects with a new mechanism of action. After this new approach, the development of antibody-drug conjugates (ADCs) did not take long, since in 1983 the first clinical trial was carried out. It is also important to mention that almost 100 years before, in 1913, Paul Ehrlich the founder of chemotherapy postulated the magic bullet concept, what meant the creation of medicines that achieve their intended cell-structure targets without damaging the normal tissues (2–5).

ANTIBODY-DRUG CONJUGATES (ADCs)

The antibody-drug conjugates consist of small molecules (payloads) covalently bound to a monoclonal antibody via a chemical linker (Fig. 1) and are designed to deliver a cell-killing agent (the payload) to a targeted cell, while limiting the systemic toxicity (4). Most of them has been created as anticancer therapy, but there are few in study phase destined to treat intracellular bacterial infection or act as anti-inflammatories (6,7).

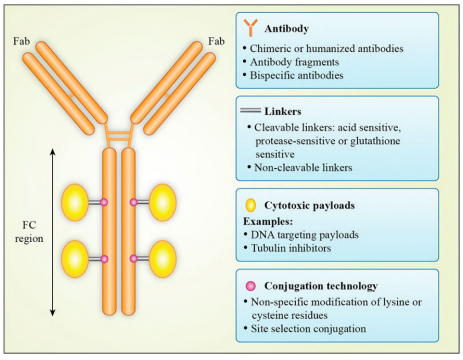

Figure 1. Structure of the antibody-drug conjugates.

In 2000, the first ADC was approved by the Food and Drug Administration (FDA) (gentozumab-ozogamicin), but in 2010 was withdrawn because the post-approval studies failed. After this, in 2011 the brentuximab-vedotin was the second approved ADCs, both of them for non-solid tumors. Finally, in 2013 the FDA approved the first ADC against a solid tumor, the ado-trastuzumab-emtansine for patients with breast cancer. After this, much more ADCs have been developed and nowadays the FDA has approved 11 and the European Medicines Agency (EMA) 7; 6 of them (approved by both agencies) are collected in Table 1 along with their parts types and disease indication (3,8).

Table 1. Parts, drug-antibody ratio (DAR), indication and year of approval for 6 ADCs approved by the FDA and EMA.

ADC	Target	mAb	Linker	Payload/ Payload Class	Payload Action	DAR	Disease Indication (Year of Approval)
Mylotarg® (gem-tuzumab ozogamicin)	CD33	IgG4	acid cleavable	ozogamicin/ calicheam-icin	DNA cleavage	2–3	CD33+ R/R AML (2000) [a]
Adcetris® (brentux-imab vedotin)	CD30	IgG1	enzyme cleavable	MMAE/ auristatin	microtubule inhibitor	4	R/R sALCL or cHL (2011)R/R pcALCL or CD30+ MF (2017); cHL, sALCL or CD30+ PTCL (2018) [b]
Kadcyla® (ado-trastuzumab emtansine)	HER2	IgG1	non-cleavable	DM1/ may-tansinoid	microtubule inhibitor	3.5	HER2+ metastatic breast cancer previously treated with trastuzumab & a taxane (2013); HER2+ early breast cancer after neoadjuvant taxane & trastuzumab-based treatment (2019)
Besponsa® (inotuzumab ozogamicin)	CD22	IgG4	acid cleavable	ozogamicin/ calicheam-icin	DNA cleavage	6	R/R B-ALL (2017)
Polivy® (po-latuzumab vedotin-piiq)	CD79b	IgG1	enzyme cleavable	MMAE/ auristatin	microtubule inhibitor	3.5	R/R DLBCL (2019) [c,d]
Blenrep® (belantamab mafodotin-blmf)	BCMA	IgG1	non-cleavable	MMAF/ auristatin	microtubule inhibitor	4	R/R multiple myeloma after at least 4 prior therapies including an anti-CD38 mAb, a proteasome inhibitor, and an immunomodulatory agent (2020) [d]

[a] As a single agent or in combination with daunorubicin and cytarabine. Mylotarg® was withdrawn from the market in 2010 and reapproved in 2017 for newly diagnosed R/R CD33-positive AML. [b] In combination with cyclophosphamide, doxorubicin, and prednisone for newly diagnosed sALCL or CD30+ PTCL and in combination with doxorubicin, vinblastine, and dacarbazine for newly diagnosed cHL. [c] In combination with bendamustine and rituximab. [d] Indication approved under accelerated approval.

COMPOSITION OF THE ADCs

As it is already said, the ADCs consist of three different parts that has to be optimized to accomplish its function.

The antibody used is predominantly monoclonal (mAb) with two main characteristics to be chosen: high specificity and high affinity to the target. Additionally, it is preferable to find an antibody with low immunogenicity, long half-life during circulation, the lowest cross-reactivity possible and be able to promote the

internalization via the receptor-mediated endocytosis. Most of the ADCs use an IgG subtype antibody across its history, although the use of Fc-silent mAbs has been increased recently (9–11).

The payload is an antineoplastic drug or a cytotoxin with high potency. The plasma stability, small molecular weight, low immunogenicity and long half-life are desirable features for the payloads. The most frequently used are microtubule disruptors and DNA-damaging agents with a high cytotoxic potential, that if used unconjugated produce an unacceptable systemic toxicity (3,4,6,9,10).

The linker should be a chemical substance capable of being stable in the blood, but being disrupted once the conjugated has entered the cell. The linker is an important part of the ADCs because it plays an important role in their pharmacokinetics and pharmacodynamics properties. There are two main types, the cleavable and the non-cleavable. The first ones depend on the physiological conditions of the cell to free the payload and are acid and protease sensitive with low blood stability, and glutathione sensitive that are blood stable and are only cleaved inside the tumor cell, where there is a high concentration of glutathione. The non-cleavable ones (thioether bound) form non-reducible bound with the antibody so they have longer half-life and are more stable in blood. All the ligands have to be attached to the Ab in the constant fragment since if they are attached to the variable region may block the recognition site. Some examples of the main linker structures are hydrazone, disulfide, peptide and thioether (Fig. 2) (6,9,10,12).

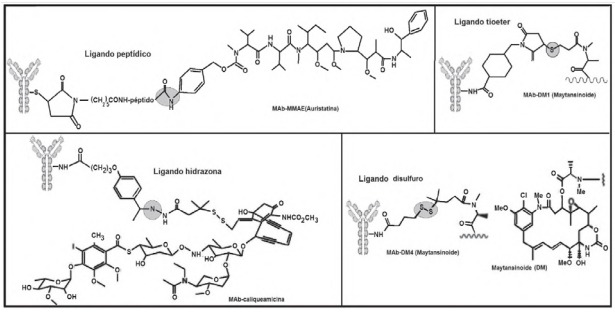

Figure 2. Main linker structures used in ADCs.

Apart from these components, it is also very important the selection of the target antigen as to accomplish the safety and efficacy of the ADCs. Some needed characteristics are the abundance of the antigen in the tumor cell compared with the normal cells, low heterogeneity across the tumor, minimal secretion to circulation and the most important property is the need to have an adequate and high efficiency internalization ability, even after binding with the ADC, as the internalization is the first step of the mechanism of action of the ADCs (10,11). In Table 2 are collected different types of target antigens studied for different cancers.

As the biomarker used as the target site of the tumor cell can be shared for different tumors, Table 2, exists the possibility of the use of one ADC for different treatments. Taking this fact in consideration, a future perspective is the development of antigen maps of cancers, what will lead to the identification of most likely tumors to benefit from particular conjugates (4).

Table 2. Tumor type indication and biomarkers used as target antigens.

Indications	Target antigen
Acute myeloid leukemia	CD25, CD23, FLT3
Breast cancer	CD25, CD174, CD228, HER2, HER3, IGF-1R
Lung cancer	CD25, CD228, HER3, FAP, IGF-1R, EGFR
Gastric cancer	CD25, CD228, FLOR1

Despite the primary purpose of the ADC therapy is reducing the side effects generated by the systemic chemotherapy, this objective has been only achieved partially. Several reasons like the free circulating payload (cytotoxic agent), that produces off-target toxicity due to a high drug antibody ratio (DAR), type of linker (unspecific cleavable) or the structural differences in the molecule can explain some side effects. But not only free payload is the cause, as the conjugated agents can produce on-target off-tumor toxicity, due to the target antigen expression in other tissues, or the presence of underlying diseases, previous treatments or genomics of each patient (4). These factors must be taken into account when establishing the safety and use of these therapies.

In order to solve the free payload off-site toxicity, a new generation of ADCs with better site-specific conjugation of the payload and uniform DAR for a more controlled delivery of the cytotoxic drug is the new challenge. Some of the strategies proposed are engineered cysteines, synthetic amino acids and transpeptidases, while the off-tumor toxicities can be minimized by the pharmacogenomics (4,7,11).

MECHANISM OF ACTION OFADCs

The simplest definition of ADCs mechanism of action could be a pharmaceutical Trojan horse mechanism (4), and this is due to the unique design of the ADCs. However, how it really acts is due to the monoclonal antibodies are designed to recognize, via their hypervariable region (paratope), a specific epitope of a biomarker expressed in the membrane of a tumor cell. These epitopes are usually over expressed receptors (9).

Once the union has occurred the ADC is internalized via endocytosis with the formation of a clathrin-coated early endosome that will later fuse with a lysosome full of enzymes. Once in the endosome the release of the cytotoxic compound will occur. The cleavable linkers will disrupt in the endosome early stages, while the no-cleavable will need the enzymes from the lysosome (3,9,10).

After the cleavage, the payload is released to the cytoplasm where can mediate its action by microtubule disruption (auristatins and maytansines) or DNA-damage (duocarymycins, calicheamicins and PBD dimers and α-amanitin), resulting in apoptosis or other types of cell death like the activation of the complement system via the antibody dependent cellular toxicity (3,10,12,13). In Figure 3 a scheme of the mechanism of action of ADCs is showed.

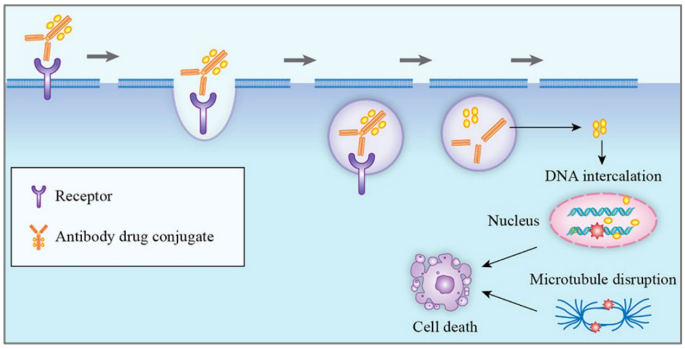

Figure 3. Mechanism of action of the ADCs.

ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF ADCs

Bioanalysis of ADCs requires multiple assays that can selectively characterize and quantify individual components that make up ADCs, what means quantification of total antibody (all ADC-DAR species), antibody conjugates and unconjugated drug (4,7).

CTI Laboratory Services in collaboration with FARMARTEM group at the University of the Basque Country (UPV/EHU) are involved in the development of the required analytical methodologies for the analysis of a new ADC to evaluate its pharmacokinetic profile in blood samples of clinical trial patients.

For the quantification of the ADCs, two parts can be differentiated and measured, the large-molecules (antibodies) and the small-molecules (payloads).

The antibodies are large molecules with a well-defined tertiary structure, so are suitable for ligand-binding assays (LBAs). The LBAs are based on the binding affinity between two molecules. The ELISA (enzyme-linked immunosorbent assay) is the most common LBAs used and the best option for the analysis of large molecules in complex biological samples. ELISA is "any antibody- or antigen- coated solid phase immunoassay with an enzyme involved in the signal generation process" with a basic procedure that involves plate coating, blocking and signal generation before the measurement; Figure 4. The most critical part of the ELISA development is the selection of the reagents: the antibody or antigen to coat the plate with (capture molecule); and the detection and signal generation antibody since the binding affinity between the molecules (antigen–antibody) are the key characteristics for the LBA sensitivity, precision and robustness (6,14,15).

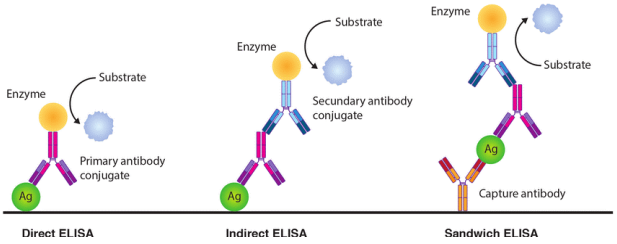

Figure 4. Types of ELISA.

On the other hand, the predominant technique for the bioanalysis of small-molecules of ADCs is the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) after the analyte extraction from the matrix (15).

This technique has been established as the most sensitive and selective analytical technique for biological samples analysis. The major advantage are the many options for adjusting LC conditions, MS/MS parameters and sample preparation protocols. Also, the use of isotopic analogs like 13C or 15N labelled standards (6,16) as internal standards improve precision and it allows the response of a given analyte to be normalized, compensating possible variations of analyte concentration during sample preparation, injection or variability in the analysis due to the equipment (6,16,17).

In the case of ADCs, the free payload can be measured by LC-MS/MS, previous extraction step (protein precipitation, SLE, LLE…) (Fig. 5). Even the payload should not be as a free molecule in the samples, it can be found due to its release during the circulation or the catabolism that may suffer the ADCs. Because of this, the main challenge is to obtain a method with enough sensitivity to detect the predictable small concentration of this free payload present on the samples (6,15).

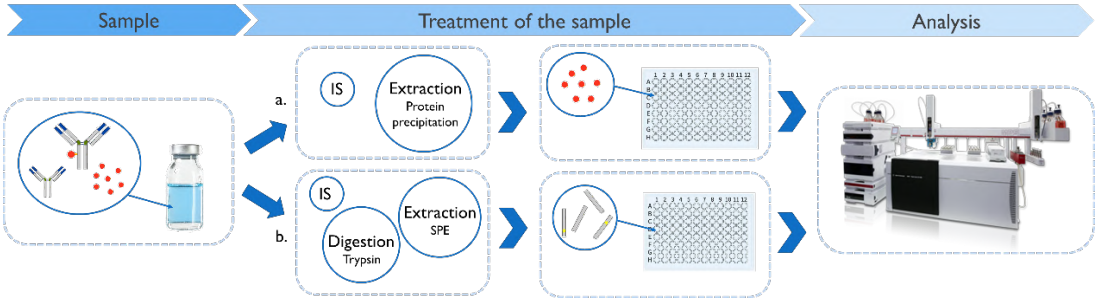

Figure 5. Analytical process for the bioanalysis of a) the free payload and b) the total amount of antibody.

In addition, the total amount of antibodies is represented by the un-conjugated antibody and the drug conjugated antibody and despite being a large-molecule, the quantitative analysis with LC-MS/MS can be carried out by performing an enzymatic digestion with trypsin to detect the signature peptide after a SPE extraction in our particular case. The signature peptide is mostly obtained from the constant region in the case of animal samples and from the variable region in humans to avoid the interferences with endogenous human immunoglobulins (6). The procedure followed to quantify by LC-MS/MS a large-molecule, like an antibody is represented in Figure 5.

As a conclusion, it can be said that a combination of different analytical techniques is required to determine the different parts of the ADCs in order to obtain a reliable information of these complex molecules in biological matrixes (15).

Until the date we have already developed and validated the LC-MS/MS method for the quantification of the free payload and the total antibody. The ELISA method is on the validation process and we expect to receive the blood samples as soon as the clinical trial starts. After the analysis of the blood samples, the pharmacokinetic profile will be generated by the data analysis of the results obtained in the bioanalysis.

In addition to the previously described it is important to mention that the whole development and analysis has to be performed following the GLP and GCP statements and the bioanalytical methods have to be validated according to the FDA and EMA guidelines. This is because this project is related to a clinical trial of an ADC intended to achieve the market access.

Bibliography:

1. Cancer [Internet]. [cited 2022 Aug 11]. Available from: https://www.who.int/news-room/fact-sheets/detail/cancer
2. Strebhardt K, Ullrich A. Paul Ehrlich's magic bullet concept: 100 years of progress. Nature reviews Cancer. 2008 Jun;8(6):473–80.
3. Tong JTW, Harris PWR, Brimble MA, Kavianinia I. An Insight into FDA Approved Antibody-Drug Conjugates for Cancer Therapy. Molecules. 2021;26(19):5847.
4. Tarantino P, Carmagnani Pestana R, Corti C, Modi S, Bardia A, Tolaney SM, et al. Antibody–drug conjugates: Smart chemotherapy delivery across tumor histologies. CA Cancer J Clin. 2021;72(2):165–82.
5. Topin I. Monoclonal antibodies – all you need to know about antibody generation | Tebubio's blog [Internet]. Tebubio. 2018 [cited 2022 May 9]. Available from: https://www.tebu-bio.com/blog/monoclonal-antibodies-all-you-need-to-know-about-antibody-generation/
6. Cahuzac H, Devel L. Analytical methods for the detection and quantification of adcs in biological matrices. Pharmaceuticals. 2020;13(462):1–16.
7. Hyung SJ, Li D, Koppada N, Kaur S, Saad OM. Method development of a novel PK assay for antibody-conjugated drug measurement of ADCs using peptide-linker drug analyte. Anal Bioanal Chem. 2019 May 19;411(12):2587–96.
8. Resources for Information | Approved Drugs | FDA [Internet]. [cited 2022 Aug 11]. Available from: https://www.fda.gov/drugs/drug-approvals-and-databases/resources-information-approved-drugs
9. Baltazar HE, Christen, González J JGC. Conjugados Anticuerpo-Fármaco: El estado de arte Antibody-Drug Conjugate: State of the Art. Rev Mej ciencias Farm. 2011;42(3):7–16.
10. Hafeez U, Parakh S, Gan HK, Scott AM. Antibody-drug conjugates for cancer therapy. Molecules. 2020 Oct 1;25(20):4764.
11. Lucas AT, Price LSL, Schorzman AN, Storrie M, Piscitelli JA, Razo J, et al. Factors Affecting the Pharmacology of Antibody–Drug Conjugates. Antibodies. 2018 Feb 7;7(1):10.
12. Kostova V, Désos P, Starck J-B, Kotschy A. The Chemistry Behind ADCs. Pharmaceuticals. 2021;14(5):442.
13. Anticuerpos monoclonales y sus efectos secundarios [Internet]. [cited 2022 May 11]. Available from: https://www.cancer.org/es/tratamiento/tratamientos-y-efectos-secundarios/tipos-de-tratamiento/inmunoterapia/anticuerpos-monoclonales.html
14. Wild D. The Immunoassay Handbook. 4th ed. Wild D, John R, Sheehan C, Binder S, He J, editors. Elsevier; 2013.
15. Kaur S, Xu K, Saad OM, Dere RC, Carrasco-Triguero M. Bioanalytical assay strategies for the development of antibody-drug conjugate biotherapeutics. Bioanalysis. 2013;5(2):201–26.
16. Volmer DA, Jessome LL. Ion Suppression: A Major Concern in Mass Spectrometry. LGLC North America. 2006;24(5):498–510.
17. Stone J. Sample preparation techniques for mass spectrometry in the clinical laboratory. In: Nair H, Clarke W, editors. Mass Spectrometry for the Clinical Laboratory. Academic Press; 2017. p. 37–62.