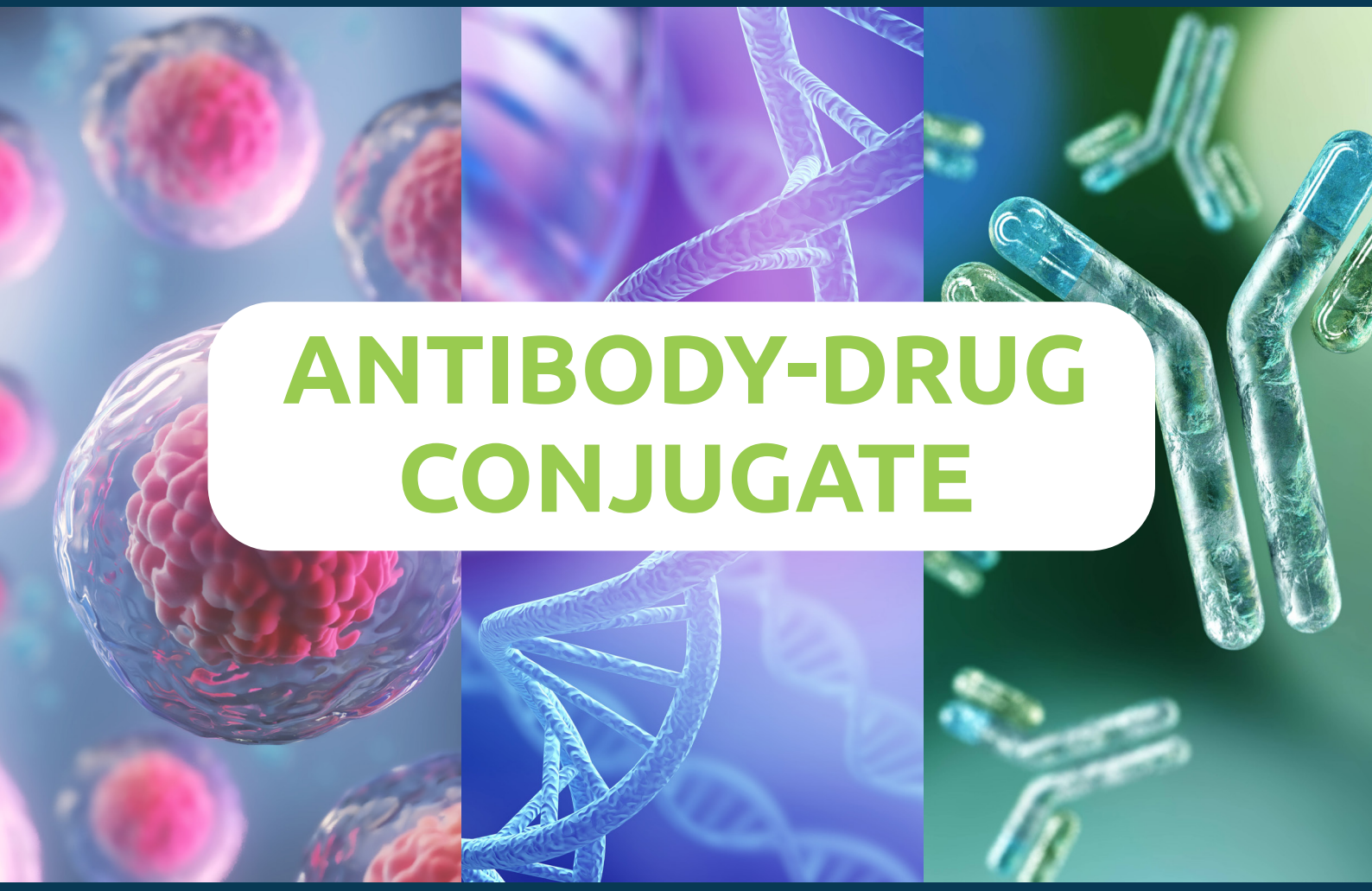
IMMUNOWATCH

EDITION N°6 - AVRIL 2023



ANTIBODY-DRUG CONJUGATE

mabdesign
THE BIOTHERAPY EXPERT

INTRODUCTION

MabDesign's Immunowatch is a one-of-a-kind information monitoring newsletter in the field of biologics. Its aim is to provide members of our association with the most recent and pertinent data gathered or generated through the key expertise of MabDesign and its collaborators in scientific research, business intelligence, market analysis and intellectual property.

Each edition will focus on trending type of biologics. Its general format includes market study research, financial and economic data, invited contributions from scientific teams working in the industry or in academia and a section dedicated to intellectual property. The content of each edition is decided by an editorial composed of two field experts. Decision concerning the theme and conception of each newsletter is done in-house by the permanent members of our editorial team.

Finally, we would like to acknowledge the support of the Ambition Recherche & Développement (ARD) Biomédicaments 2020 Phase II programme, funded by the Centre Val de Loire region during the initial phases of launching this newsletter.

BIOPHARMACEUTICALS
Innovation Dynamics in Health
IN REGION CENTRE-VAL DE LOIRE

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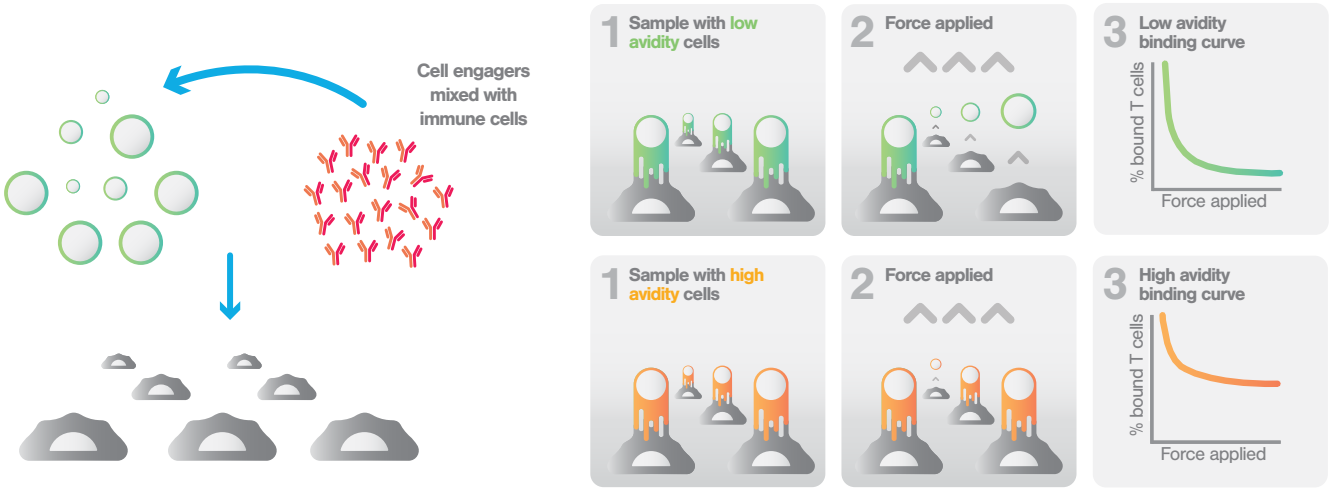
THE BIOTHERAPY EXPERT

Région Centre-Val de Loire

Separate your cell engager constructs fast and simple

Develop highly specific cell engager constructs with improved function and safety by understanding target-effector cell avidity



A novel and reproducible approach to rank cell engagers by quantifying cell-to-cell binding events

Researchers are searching for the **most effective cell engager** against cancer using an array of *in vitro* assays and affinity measurements. However, these measurements are time-consuming and do not always correlate with functionality. **What seems to be the best candidate now might turn out to be the wrong one later.**

Cell avidity analysis measures the **total intercellular strength** between interacting cells and allows researchers to separate and rank their cell engager constructs based on synaptic strength. This provides a **novel biomarker** to characterize the key attributes in the formation of a strong immune synapse and ultimately identify **the cell engager that efficiently improves tumor cell killing**.

Discover the z-Movi® Cell Avidity Analyzer

Find out how cell avidity can take your research further

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EDITORIAL

Jean-Guillaume Lafay

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We are honored to be co-editors of this issue of Immunowatch dedicated to new antibody formats. Mablink is an emerging actor in the antibody-drug conjugates (ADC) field, which develops oncology-focused ADCs based on its proprietary hydrophilic ADC platform: PSARlink™. As such, we are witnessing first-hand the intense emulation created by the recent successes of ADCs such as Daiichi Sankyo's Enhertu™. Companies are created almost on a weekly basis, all over the world, to develop new ADCs based on innovative linkers or payloads or using new antibody formats such as bispecific, biparatopic or conditionally active antibodies. Big Pharma is also coming back to the field with a slew of deals to acquire ADCs or ADC technologies. We even start to see an expansion outside of the oncology field, for example with the ABBV-3373 combining the adalimumab and an anti-inflammatory payload.

As a company with a lead ADC 12 months away from the clinic and a proprietary platform technology, we get to meet regularly with prospective investors and potential biotech or pharma partners: the potential of these new antibody formats is clear to everyone! It is now our role, as a field, to transform these innovations, of which this edition is providing a great overview, into new treatments, for the greater good of the patients and their families.

The design of innovative anticancer targeted therapies with superior antitumor efficacy and reduced toxicity continues to be a challenging endeavor. More than a century ago, Paul Ehrlich came up with an original concept known as "the magic bullet". His vision was to link a cytotoxic payload to an entity with selective affinity for a tumor, with the aim of achieving the selective delivery of chemotherapeutic agents in the tumor environment and reducing systemic toxicity. Although this concept seems to be quite simple, its fine-tuning is fairly elaborate and still challenging today. Over the past five decades, the advances of antibody–drug conjugates (ADCs) made them one of the best embodiment of Paul Ehrlich's "magic bullet" vision, as a vectorized targeted therapy, harnessing both the high potency of the drug against the malignancy and the high specificity of the antibody for its target. New strategies in development participate to their perpetual improvement, through innovations brought to the linker (especially the release payload mechanism and its hydrophobicity profile), the target or the format of the antibody, the ADC PK-PD properties and mechanism of action. These improvements promise a bright future for this exciting area of research, and allow ADCs to get a little closer to the magic bullet imagined by Paul Ehrlich, for applications in oncology and beyond.

GLOBAL ADC market

Discover the marketed products, pipeline drug candidates, major deals and biopharmaceutical companies

ADC market

ADC sales netted 7,4 billions US$ in 2022 which represent 3% of antibody global sales.

Number of drugs on market ADC vs monoclonal Ab

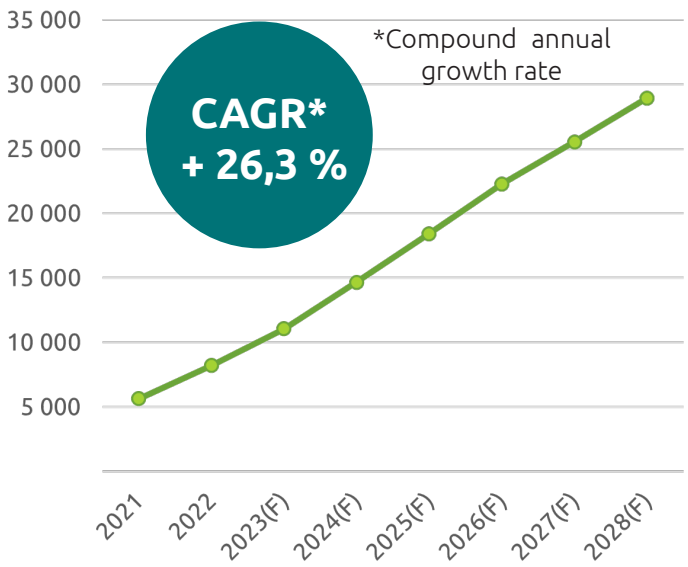
Marketed ADC indication by tumor type

18 mAb conjugated marketed only in oncology field in more than 29 indications

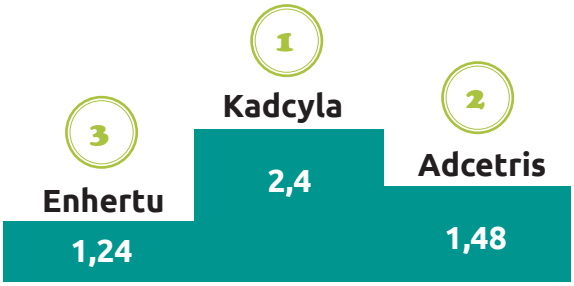
Main ADC Actors

Daiichi-Sankyo, Roche, Seagen, Gilead, abbvie, Takeda, Pfizer, astellas

Global ADC Sales and Forecast 2021-2028 (millions US$)



Top 3 ADC product sales in 2022 (in US$ billion)



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ADC market

Characteristics of marketed ADC-products

Brand Name	Drug name	mAb target	Payload	Cleavable
Adcetris	Brentuximab vedotin	CD30	Monomethyl Auristatin E (MMAE)	yes
Aidexi	Disitamab vedotin	Epidermal growth factor receptor 2 (HER2)	Monomethyl Auristatin E (MMAE)	yes
Besponsa	Inotuzumab ozogamicin	CD22	N-acetyl-gamma-calicheamicin dimethylhydra-zide	yes
Blenrep	Belantamab mafodotin	CD38	Monomethyl auristatin phenylalanine (MMAF)	no
Elahere	Mirvetuximab Soravtansine	Folate receptor 1 (FOLR1)	Maytansinoid DM4	yes
Enhertu	Trastuzumab deruxtecan	Epidermal growth factor receptor 2 (HER2)	Derivative of the camptothecin analog exate-can (DXd)	yes
Kadcyla	Trastuzumab emtansine	Epidermal growth factor receptor 2 (HER2)	Maytansinoid DM1	no
Mylotarg	Gemtuzumab ozogamicin	CD33	Calicheamicin	yes
Padcev	Enfortumab vedotin	Nectin 4	Monomethyl Auristatin E (MMAE)	yes
Polivy/ RoPolivy	Polatuzumab vedotin-piiq	CD79b	Monomethyl Auristatin E (MMAE)	yes
Tivdak	Tisotumab vedotin	CD142 (Tissue Factor or Thromboplastin)	Monomethyl Auristatin E (MMAE)	yes
Trodelvy	Sacituzumab govitecan-hziy	Trop-2 (the tro-phoblast cell-surface antigen-2)	Drug SN-38	yes
Ujvira	Trastuzumab emtansine biosimilar (Ujvira)	Epidermal growth factor receptor 2 (HER2)	Maytansinoid DM1	no
Zynlonta	Loncastuximab Tesirine	CD19	Pyrrolobenzodiazepines (PBD)	yes

Characteristics of marketed other antibody conjugates

Brand Name	Drug name	mAb target	Payload	Cleavable	Conjugates type
Akalux	Cetuximab sarotalocan	Epidermal growth factor receptor (EGFR)	Phthalocyanine dye IRDye 700DX	no	Antibody Light-activatable dye Conjugate
Licartin	iodine I 131 metuximab	CD147	Iodine 131	no	Radio-Immunoconjugates
Zevalin	Ibritumomab tiuxetan	CD20	Yttrium-90	no	Radio-Immunoconjugates

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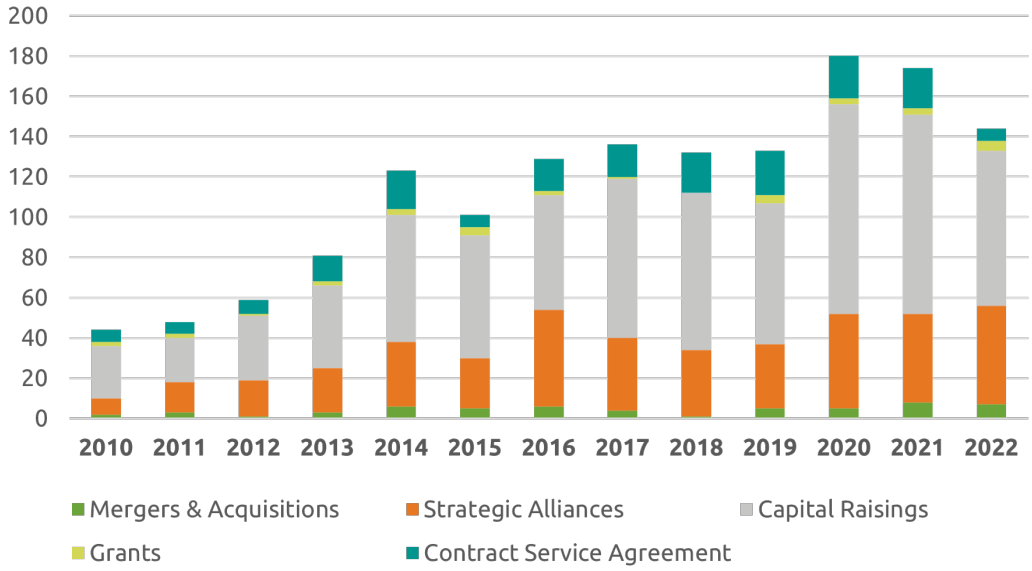
ADC deals

Number of deal by type and year



ADC deal value in 2021

In 2021

140
Acquisition (millions US$)

16
Strategic Alliances (billion US$)

5.6
Capital Raisings (billion US$)

Recent deals in ADC field

Partners		Deal value	Deal description
Pfizer	Seagen	43 (billions US$)	Pfizer to Acquire Seagen for USD43 Billion
Gilead Science	Immunomedics	21 (billions US$)	Gilead Sciences Acquires Immunomedics
Bristol Myers Squibb	Eisai	3,3 (billions US$)	Eisai enters into co development agreement with Bristol Myers Squibb
ImmunoGen	Lilly	1,7 (billions US$)	ImmunoGen Announces a Global, Multi-Target License Agreement of its Novel Camptothecin ADC Platform to Lilly
Mersana Therapeutics	Janssen	$40 million US$ upfront payment and potentially more than $1 billion in total milestones	Mersana Therapeutics Announces Research Collaboration and License Agreement with Janssen to Advance Novel Antibody-Drug Conjugates

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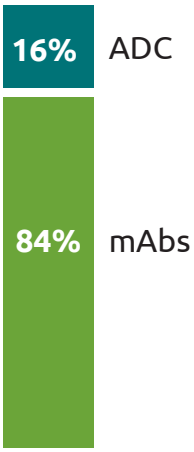
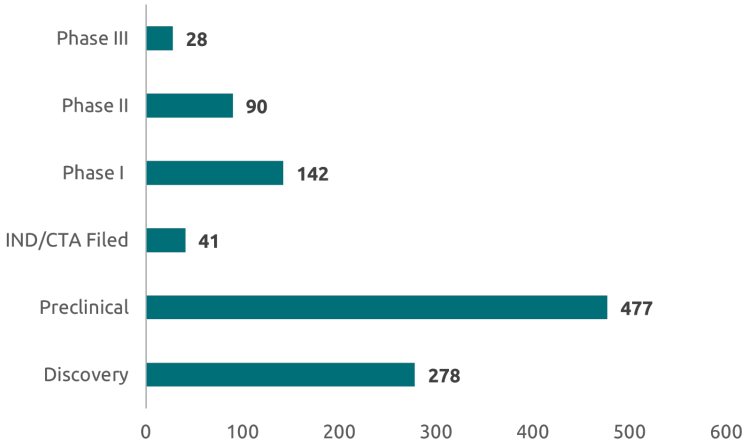
ADC clinical trials

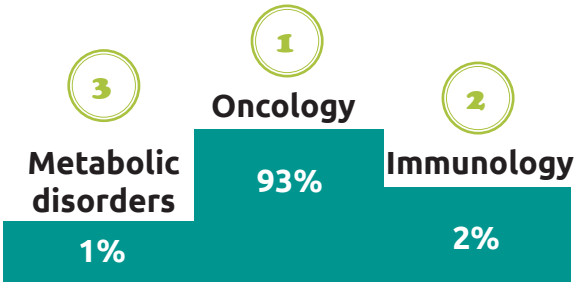
ADC vs other Ab in development



ADC pipeline phase distribution



Top 3 Therapeutic areas



New ADC products in Phase III and pre-registration in oncology by tumor type

Characteristics of new ADC in phase III and pre registration in oncology

Drug name	mAb target	Payload	Cleavable
trastuzumab duocarmazine	HER-2	Duocarmycin	yes
ARX-788	HER-2	Amberstatin 269	no
BAT-8001	HER-2	3AA-MDC (a Maytansine derivative)	no
datopotamab deruxtecan	Trophoblast cell-surface antigen 2	Exatecan derivative, DXd	yes
patritumab deruxtecan	HER-3	Exatecan derivative, DXd	yes
SHRA-1811	HER-2	Rezetecan	yes
SKB-264	Trophoblast cell-surface antigen 2	Belotecan-derivative	yes
TAA-013	HER-2	Maytansinoid DM1	yes
Telisotuzumab vedotin	Hepatocyte growth factor receptor	Monomethyl auristatin E (MMAE)	yes
tusamitamab ravtansine	CEA CAM5 or CD66e	Maytansinoid DM4	yes
upifitamab rilsodotin	NaPi2b	Auristatin	yes
vobramitamab duocarmazine	CD276	Duocarmycin	yes
zilovertamab vedotin	ROR1	Monomethyl auristatin E (MMAE)	yes

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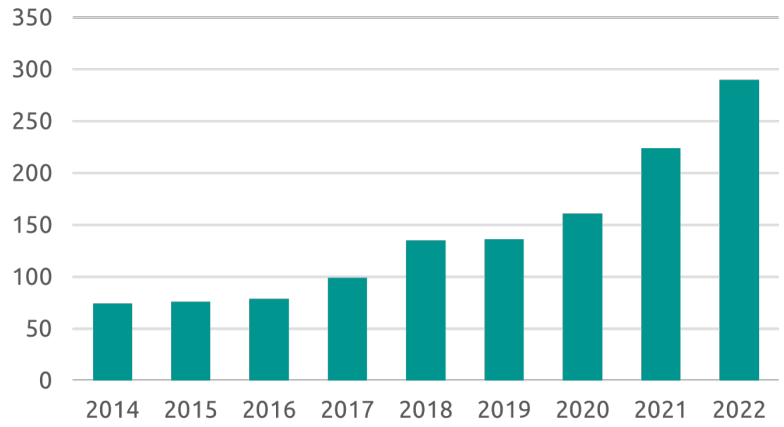
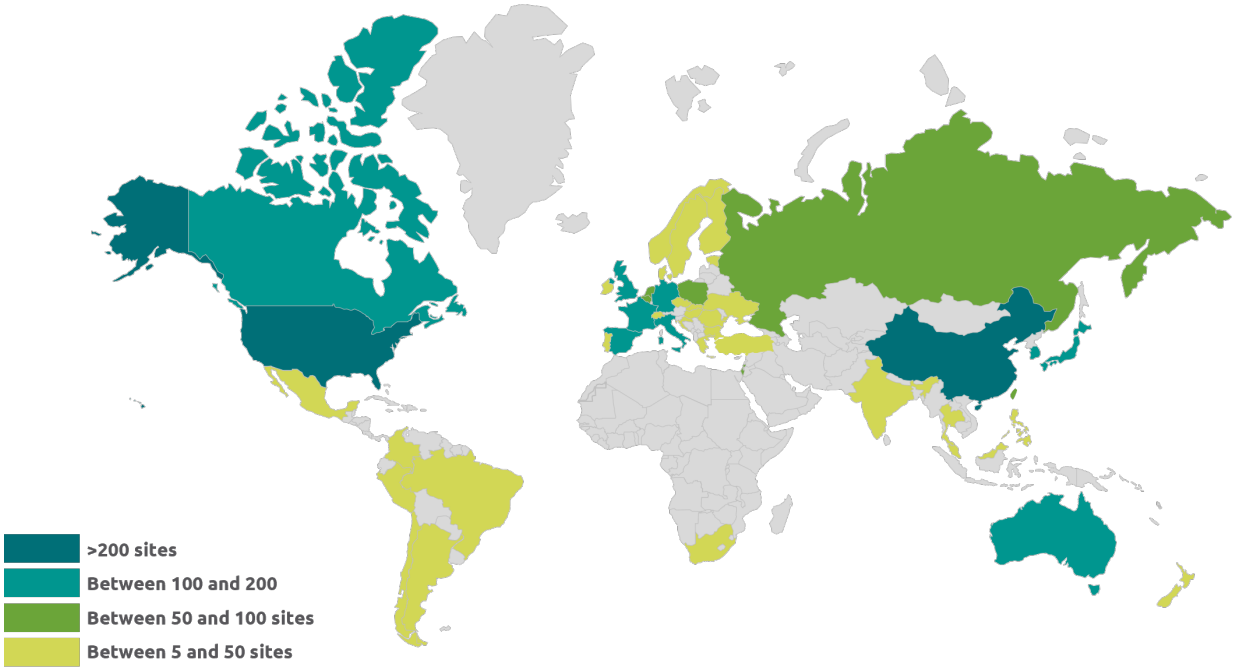
ADC clinical trials

Number of clinical trials for ADC by start year



Distribution of clinical trial sites for ADC



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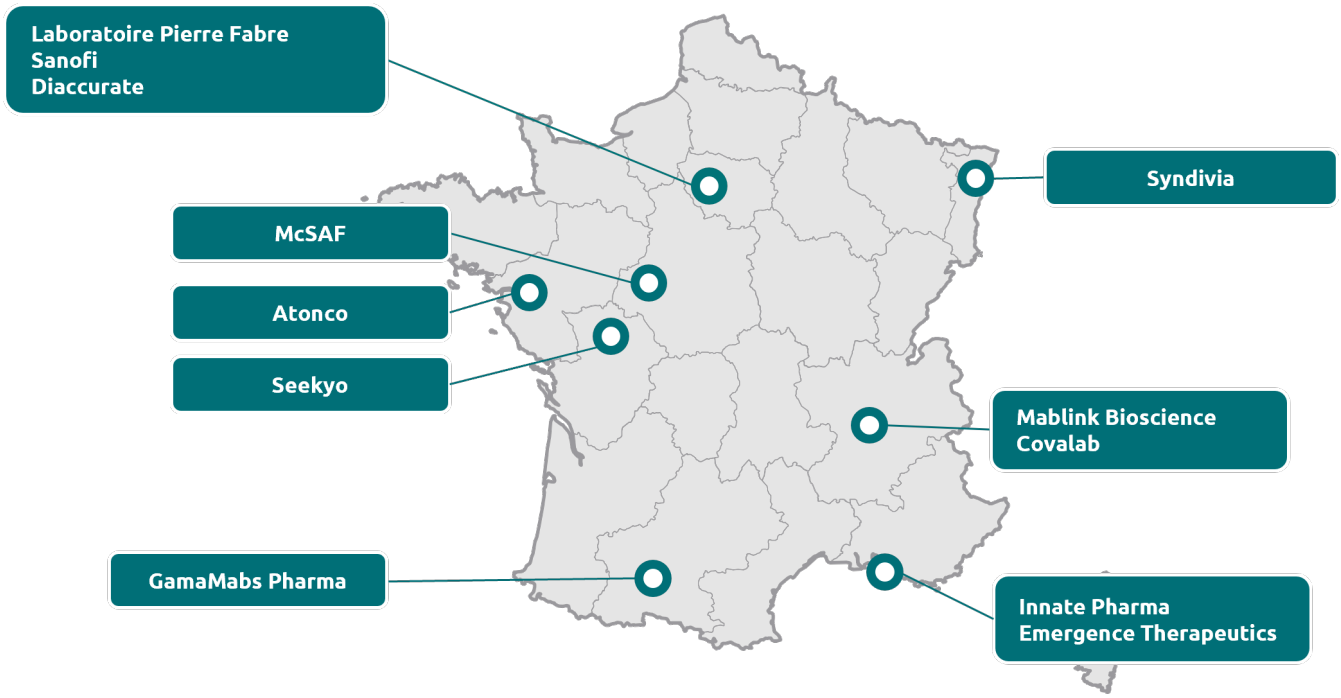
ADC in France

ADC developers in France



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scientific articles

Read the different inputs from the scientific community on various aspects of ADC

LONIGUTAMAB UGODOTIN (W0101) ANTIBODY DRUG CONJUGATE: CHEMISTRY MANUFACTURING AND CONTROLS

*Jean-François Haeuw [1], Elsa Wagner [1], El Bachir Kaloun [2], Stéphane Chenu [1,4], Matthieu Culie [1,4], Sandrine Demare [1,4], Sandrine Lauthier [1,4], Thomas Ballet [2], Nancy Regent [2], Sarah Cianférani [3], and Alain Beck [1,2]**

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1. Introduction

The insulin-like growth factor type 1 receptor (IGF-1R) is involved in tumorigenesis and overexpressed in many tumors. So far, therapeutic approaches based on mAbs and tyrosine kinase inhibitors targeting IGF-1R have only shown clinical benefit in specific patient populations in oncology. IGF-1R targeted antibody drug conjugate (ADC) W0101 was designed to deliver a highly potent cytotoxic auristatin derivative selectively to IGF-1R overexpressing tumor cells (Akla B et al, 2020). The monoclonal antibody (mAb) hz208F2-4 (lonigutamab) component was selected for its specific binding properties to IGF-1R compared with the insulin receptor, and for its fast internalization properties. The conjugation of the auristatin-based drug linker (DL) ugodotin to hz208F2-4 did not alter its binding and internalization properties. W0101 induced receptor-dependent cell cytotoxicity in vitro when applied to various cell lines overexpressing IGF-1R but did not affect normal cells. Efficacy studies were conducted in several mouse models expressing different levels of IGF-1R to determine the sensitivity of the tumors to W0101.

A phase I/II open label dose escalation and dose expansion study of intravenous infusion of W0101 in patients with advanced or metastatic solid tumors was launched in December 2017 (NCT03316638, www.clinicaltrials.gov).

Here we report W0101 Chemistry, Manufacturing and Controls (CMC) workflows including the DL (F556311, ugodotin), the mAb (hz208F2-4, lonigutamab), the ADC Drug Substance (DS, lonigutamab ugodotin) and ADC Drug Product (DP, W0101) (**Figure 1**).

2. Chemistry: W0101 structure and components (Figure 1, Akla B et al, 2020).

W0101 (F50085-hz208F2-4-F556311, lonigutamab ugodotin; WHO, INN list 86, 2021) is an ADC based on mAb F50085-hz208F2-4 (specific for human Insulin-like growth factor 1 receptor (IGF-1R)) and a drug-linker F556311. The drug-linker is composed of a highly potent anti-microtubule agent derivative of mono-methyl auristatin F, coded F554443, and a non-cleavable maleimidocaproyl (mc) linker that covalently attaches F554443 to F50085-hz208F2-4 (Figure). An average of 4 drug-linker molecules are coupled per antibody molecule.

The non-cleavable maleimidocaproyl linker was selected for its stability in systemic circulation and potency of releasing the cytotoxic payload in the intracellular compartment.

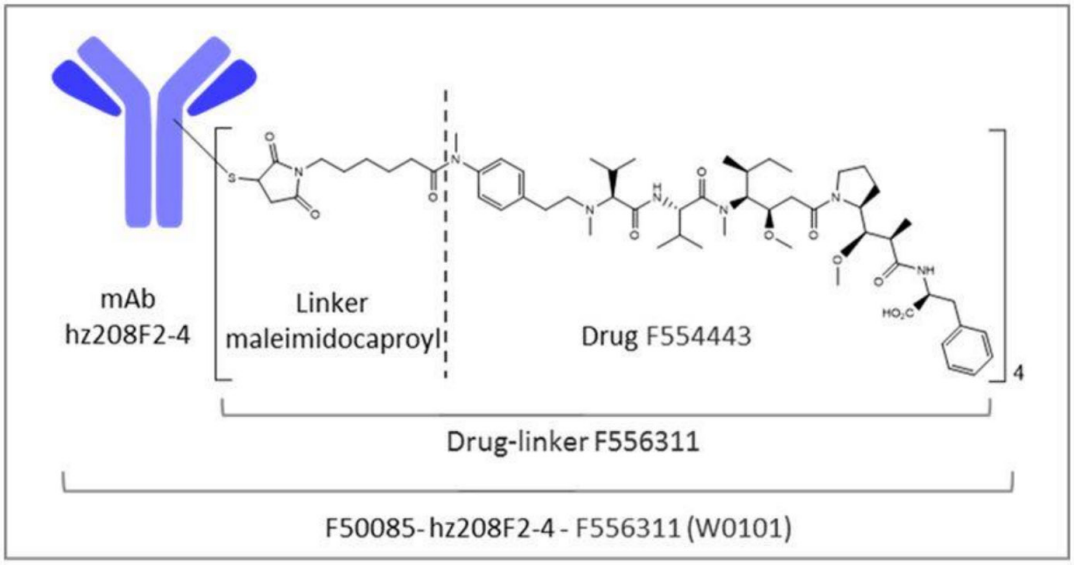


Figure 1: Structure of W0101 ADC (lonigutamab ugodotin)

3. Manufacturing

3.1. Drug Substance Intermediate (DSI): Drug-Linker F556311 (ugodotin)

Briefly, 6-Maleimidohexanoic acid is transformed to acid chloride by reaction with oxalyl chloride. The reaction mixture is stirred at room temperature, concentrated in vacuo and dried on high vacuum at room temperature. The resulting product is kept in solution in dichloromethane and will be used in the next step. F554443 is dissolved in dichloromethane and diisopropylethylamine is added at 0°C. The coupling is carried out by addition of 6- maleimidohexanoyl chloride solution (prepared freshly) on the reaction mixture at 0°C. At the end of the reaction, the reaction mixture is quenched with water and the phases are separated. The aqueous phase is extracted with dichloromethane. The combined organic phases were dried (MgSO4), filtered and concentrated in vacuo. The crude product is first purified by column chromatography on silica gel (Dichloromethane/methanol) and then by preparative HPLC (water/acetonitrile). Pure fractions are extracted with dichloromethane. The organic phase is washed, dried (MgSO4) and concentrated in vacuo. The pure product is dissolved in water/acetonitrile and lyophilized.

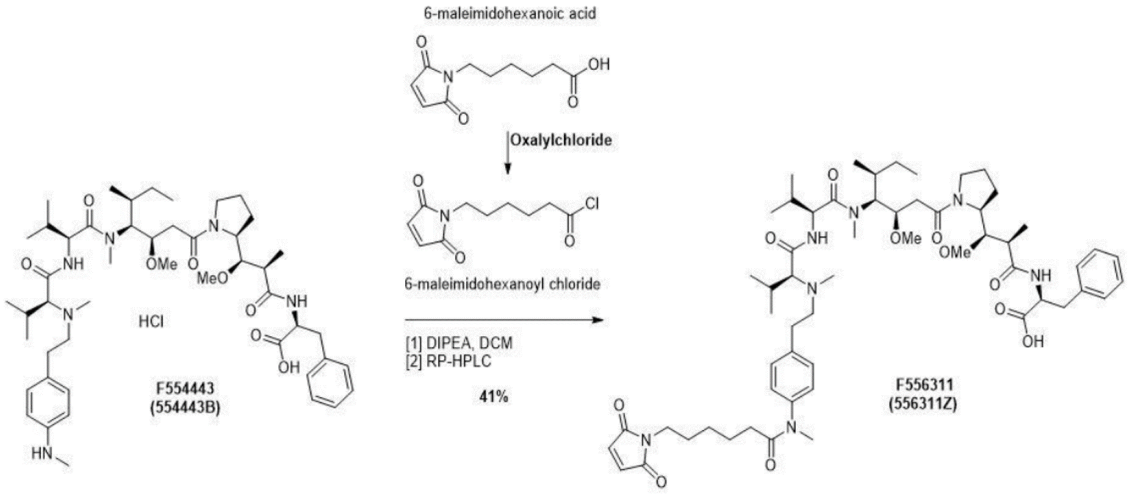


Figure 2: F556311 DL manufacturing flow chart

3.2. Drug Substance Intermediate (DSI): mAb hz208F2-4 (lonigutamab)

Manufacturing of the antibody begins with the thawing of a GMP Cell Bank vial and continues through a series of cell expansion steps, leading to cultivation in a stirred bioreactor. The content of the bioreactor is harvested, purified and filtered to produce the finished antibody. An overview diagram of these manufacturing operations is provided in **Figure 3**.

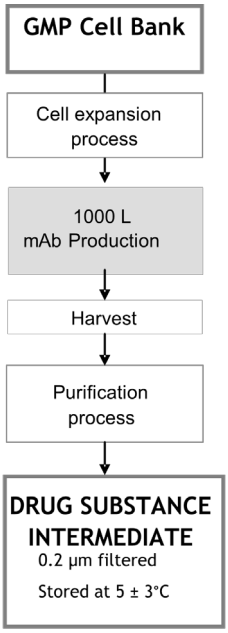


Figure 3: hz208F2-4 mAb manufacturing flow chart

Briefly, to produce each antibody batch, the CHO cell line is expanded through multiple cultivation stages up to the production bioreactor.

The clarification to remove CHO cellular biomass and associated debris is performed with self-contained depth filters. A dead-end microfilter (0.5/0.2 µm, low-protein-binding) is used in line after the depth filter to remove residual particles, to reduce the bioburden and to prepare the product stream for purification steps.

The mAb is first captured on a Protein A resin. The clarified supernatant is loaded onto the resin. The components of the culture media and most of the impurities present in the clarified product stream are mostly removed in the flow through, including DNA and host cell proteins (HCPs). After loading, a wash step removes non-specifically bound contaminants such as HCPs. The eluted product is treated to inactivate potential viruses. pH inactivated Protein A eluates are pooled together and filtered through a depth filter followed by a dead-end 0.5/0.2µm filter to reduce bioburden, DNA and HCP levels.

The product is then loaded on a strong cation exchange column. The step is performed in a bind and elute mode and further improves HCP removal and multimer removal if present.

The antibody eluted from cation exchange column is loaded on an anion exchange membrane operated in flow through mode. This final polishing step removes residual DNA, HCP as well as viruses. The mAb concentration is adjusted at the end of anion exchange step, before viral filtration.

The viral filtration train (nanofiltration) consists in a depth prefilter, a 20 nm polyethersulfone (PES) nanofilter and a 0.5/0.2µm filter.

The product is formulated by diafiltration and concentrated into the final buffer with single-use cassettes.

The final process step is a 0.5/0.2µm filtration. The antibody concentration is adjusted the bioburden is tested. The antibody solution is aseptically dispended into sterile disposable bags and stored at +5°C 3°C. This product serves as the starting material for ADC synthesis.

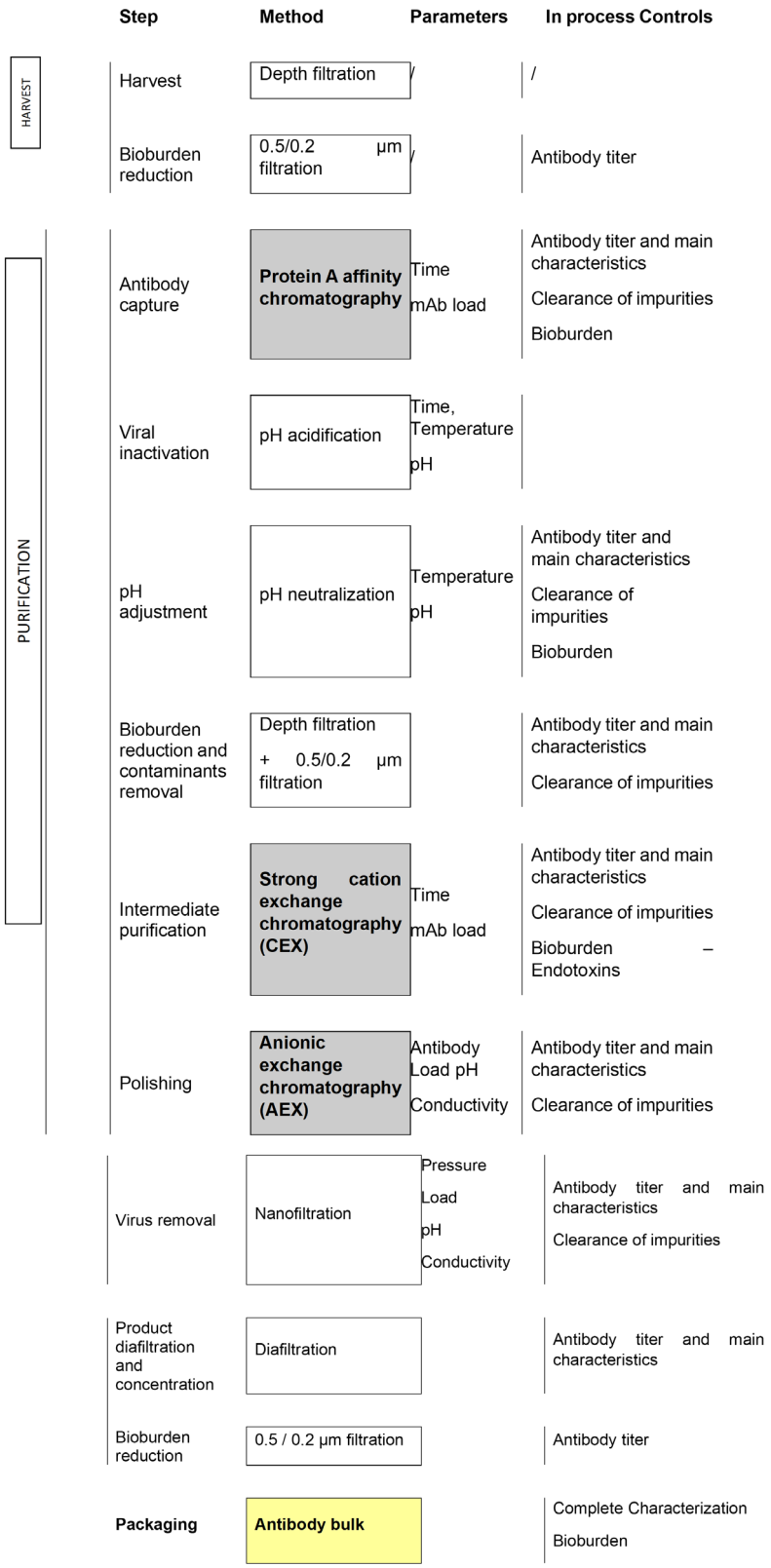


Figure 4: hz208F2-4 mAb harvest and purification

3.3. ADC Drugs Substance (DS) (lonigutamab ugodotin)

Briefly, ADC DS manufacturing begins by the solubilization of the drug-linker into DMSO followed by a filtration on 0.2µm PES filter.

The mAb is first diluted and then partially reduced by TCEP 2mM (ratio 2.75 mol TCEP/mol antibody). This step is made in a stainless-steel reactor. The aim of this reduction is to open disulfide bridges to allow the drug-linker to link the free thiol group of cysteine.

Once reduced, the mAb is diluted and then, the drug-linker is added in excess.

After coupling, the reaction is stopped by addition of a solution containing N-acetylcysteine (NAC) (ratio 1.5 mol/mol of drug-linker) which quenches the remaining drug-linker molecules.

After overnight storage at +5°C, the product is then diafiltered and concentrated into final buffer to a concentration of 5 g/L with single-use self-contained cassettes.

The final process step is a 0.5/0.2 µm filtration.

The ADC DS is then transformed in ADC DP by a sterile filtration and vial filling for clinical trials.

Step	Components	Parameters	In process Controls
1-Drug-linker solubilisation	drug-linker (F556311) DMSO		/
2-Antibody mild reduction	mAb (F50085-hz208F2-4) Borate buffer TCEP	Time, Temperature, stirring speed	Antibody purity and size repartition
3-Drug-linker – antibody coupling	Reduced mAb Solubilized drug-linker (F556311) Borate buffer DMSO Sucrose	Time, Temperature, stirring speed	/
4-Drug-linker quenching	N-acetyl cysteine (NAC)	Time, Temperature, stirring speed	Antibody titer, DAR, ADC purity, impurities, bioburden
5-ADC concentration and buffer conditioning	Histidine buffer		Antibody titer, DAR, ADC purity, impurities, bioburden
6-ADC filtration	0.5 / 0.2µm filter Histidine buffer with tween		ADC titer
7- ADC packaging	PET 5L bottles		Drug substance release test

Figure 5: Manufacturing process for the ADC drug substance

3.4. ADC Drugs Product (DP)

Fhe flow diagram of the manufacturing process (fill and finish) and process controls of W0101 solution are sumerized in **Figure 6**.

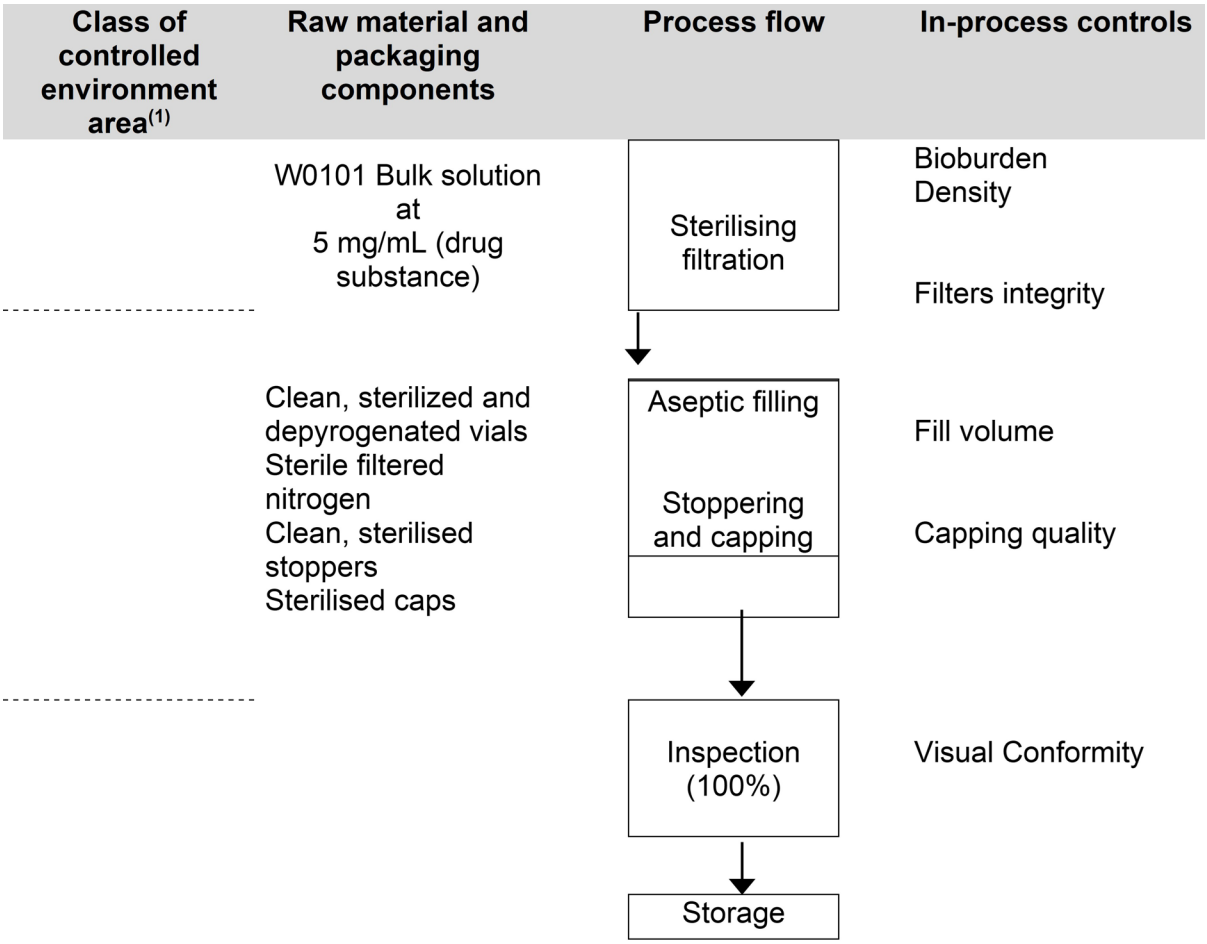


Figure 6: Flow diagram of the ADC DP Manufacturing and Process Controls

The bioburden and the density of the bulk solution is checked and sterilized by filtration through a polyether sulfone membrane filter with a pore size of 0.5/0.2µm. The filtering unit is sterilized in an autoclave prior to use. Check for the integrity of the sterilizing filter by a diffusion test implemented before and after the sterilizing filtration.

Aseptically subdivide the sterilized bulk solution into the clean sterilized and depyrogenated vials with an automatic filling and stoppering machine (vials are washed with purified water, rinsed with water for injections, sterilized and depyrogenated).

The fill volume solution is checked throughout the filling operation. The vials are stored at a temperature between 2°C and 8°C.

4. Quality controls and characterization tests

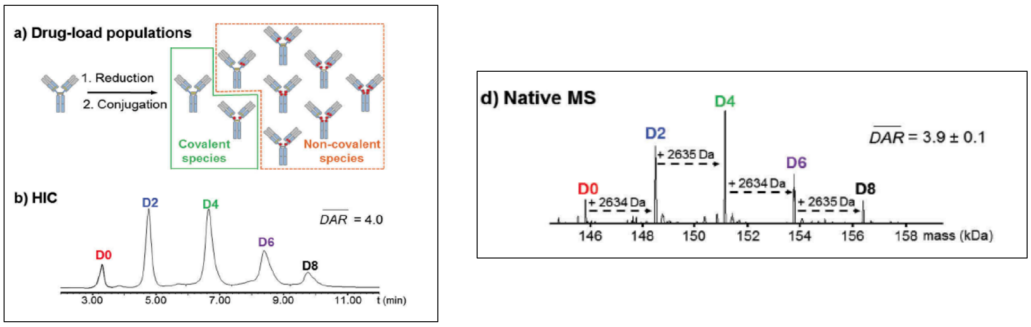
The development and optimization of ADCs rely on improving their analytical and bioanalytical characterization, by assessing critical quality attributes (CQAs). Among the CQAs, the glycoprofile, drug load distribution (DLD), the amount of unconjugated antibody (D0), the average drug-to-antibody ratio

(DAR), the drug conjugation sites and the residual drug-linker and related product proportions (SMDs) in addition to high and low molecular weight species (H/LMWS), and charge variants are the most important ones (Beck A et al, 2016; Beck A et al, 2019).

4.1. W0101 structure

In ADC W0101, two or three of the inter-chain disulfide bridges are not present and an average of 4 cysteinyl being conjugated each via a thioether bond to a drug-linker. ADC DS is a controlled mixture of D0, 2, 4, 6 and 8 species as for six other FDA, EMA or SFDA approved ADCs namely Adcetris (brentuximab vedotin, DAR4, 2011), Polivy (polatuzumab vedotin, 2019), Padcev (enfortumab vedotin, DAR 3-4) Blenrep (belantamab mafodotin, DAR4), Tivdak (tisotumab vedotin, DAR4, 2021) and Aidexi (disatamab vedotin, DAR 4, 2021, China) (Joubert N et al, 2020).

(A) Brentuximab vedotin : HIC and native MS



(B) Lonigutamab ugodotin (W0101): native MS

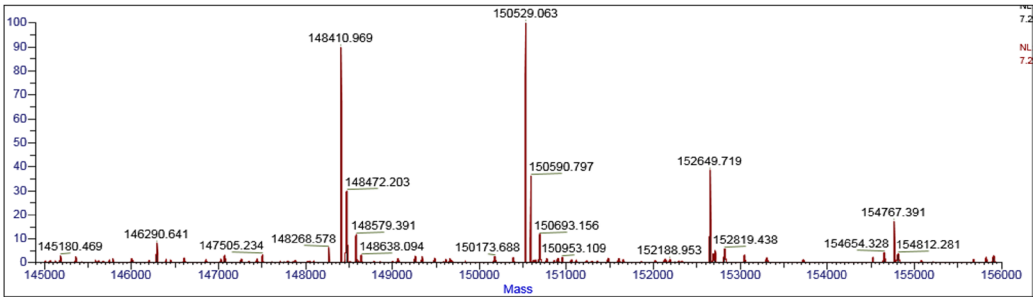


Figure 7: (A) Brentuximab vedotin : Hydrophobic Interaction Chromatography (HIC) and native Mass Spectrometry; (B) Lonigutamab ugodotin : native Mass Spectrometry

4.2. W0101 ADC DS release tests

Analytical, structural, and functional assays are used in quality control (QC) monographs for the characterization of ADCs. They may be used for drug substance/drug product batch release or characterization as well as for comparability, stability studies, and bioanalysis). The tables below show release and characterization tests used for W0101 ADC.

Release Tests (ADC DS)
APPEARANCE
IDENTITY of ADC
By Hydrophobic interaction chromatography (HIC)
Binding by ELISA (IGF1R)
Subunit profile by LC (reduced ADC)
TESTS
Color of the solution Degree of opalescence of the solution
pH
Average DAR
Target binding – EC50 ratio (in % of reference substance) by ELISA
Biological activity (cytotoxicity) – IC50 ratio (in % of reference substance)
Size variants (% area) (SEC-UV): Monomer/ Aggregates/ Fragments
Drug loading and distribution (DLD) by HIC
Residual drug and related substances by LC (Sum % w/w (drug/ADC))
Residual DMSO by LC (ppm)
Bacterial endotoxins (IU/mg ADC) (Ph. Eur. 2.6.14)
Microbiological examination by enumeration tests (CFU/10 mL) (Ph. Eur. 2.6.12) Total Aerobic Microbial Count (TAMC)/ Total Yeasts and Moulds Count (TYMC)
QUANTITY Protein content by UV (mg/mL)

4.3. W0101 ADC DS characterization tests

Total charge variants (icIEF) (%) : Basic/Main/Acidic variants
High order structure by Circular Dichroism : Far UV spectra, Alpha helices/Beta sheets (in %), Near UV spectra
Size pattern, denaturing conditions (non-reduced SDS-PAGE): HHLL/HHL/HH/HL/H/L (%)
Size pattern, denaturing condition (reduced SDS-PAGE) : Light chain (L0, L1), [MW(Da)/%]; Heavy chain (H0,
Size variants (% area) (A4F) : Monomer, Aggregates, Fragments
Free thiols (in mol of free thiols per mol of mAb)
Protein content by HPLC protein A (mg/mL)
Sub-visible particles by micro flow imaging (particles/mL) : 10µm, 25µm
Sub-visible particles by Ph. Eur. 2.9.19, Optical Microscopy (particles/mL), 10µm, 25µm
Elementary impurities by ICP-MS

5. Conclusion

Because of the hybrid nature of ADCs, product CQAs for both the biological component and the small-molecule components must be simultaneously considered. In addition, early-developability assessment requires well established but also state-of-the-art analytical and structural techniques, such as native and ion mobility mass spectrometry, but also modern to multidimensional liquid chromatography and capillary electrophoresis methods hyphenated to mass spectrometry.

W0101 ADC (lonigutamab ugotonin) GMP manufacturing is based on a robust process including 4 components (DL, mAb, ADC DS and ADC DP). The conjugation step based on limited reduction of 4 hinge interchain disulfide bridges of hIgG1k antibodies followed by DL alkylation (Sun M et al, 2005) was successfully applied to 9 out 13 FDA, EMA or SFDA approved ADCs namely Adcetris (brentuximab vedotin, DAR4, 2011), Polivy (polatuzumab vedotin, 2019), Padcev (enfortumab vedotin, DAR 3-4, Enhertu/ trastuzumab deruxtecan, DAR 8, 2019), Trodelvy (sacituzumab govitecan, DAR 7.6, 2020), Blenrep (belantamab mafodotin, DAR4), Tivdak (tisotumab vedotin, DAR4, 2021), Zynlonta (loncastuximab tesirine, DAR 2.3, 2021) and Aidexi (disatamab vedotin, DAR 4, 2021, China) (Dumontet C et al, 2023).

Mylotarg (gemtuzumab ozogamicin, DAR 2-3, 2000/2017), Kadcyla (trastuzumab emtansine, DAR 3-4, 2013), Besponsa (inotuzumab emtansine, DAR 5-6, 2017) and Elahere (mirvetuximab soravtansine, DAR 3.5-5, 2022) are based on stochastic lysine residues conjugation.

Long-term real-time ICH stability studies have been conducted for W0101 DS and DP confirming the predictions of developability assessment studies performed during the preclinical studies and the selection of the optimized clinical candidate.

Acknowledgements

To all Pierre Fabre IGF1R project teams and subteams members.

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- www.clinicaltrials.gov

THE PERPETUAL IMPROVEMENT OF ANTIBODY–DRUG CONJUGATES (ADCS)

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1. Introduction/History

Antibody–drug conjugates (ADCs) have made considerable progress in the past 50 years [1]. ADC is a vector-based targeted therapy that allows the selective delivery of a potent cytotoxic agent within a tumor. An ADC results from the stochastic or homogeneous grafting of a cytotoxic agent onto a monoclonal antibody (mAb) via a judiciously constructed spacer arm [2],[3]. This is a complex mixture of immunoconjugates with different DLD (drug loading and distribution) and DAR (drug-to-antibody ratio, corresponding to the number of cytotoxics grafted onto the mAb) [4]. In 2009 gemtuzumab ozogamicin (Mylotarg®) was the only ADC approved by the Food and Drug Administration (FDA) studies [5]. At present, 14 ADCs have been approved worldwide and more than 1685 were registered in clinical studies [6]. More than 50 candidates have also been abandoned in the clinic mainly for toxicological reasons or because of a lack of efficiency, and ADCs targeting solid tumors are currently making a satisfactory breakthrough in the clinic (until November 2019, only Kadcyla® had an indication in solid tumors).

In 2008, calicheamycins, auristatins and maytansinoids were the main classes of cytotoxics used for ADC development. Fifteen years later, these same molecules are still used among other payloads optimized for better stability and hydrophilicity. New classes of cytotoxics have also been developed (PBDs, duocarmycins and camptothecin derivatives). In 15 years, considerable progress has been made in antibody engineering to allow more site-specific conjugation, to improve the homogeneity and the stability of the constructions and to bring 2nd and 3rd generation ADCs to the clinic in the hope to broaden the therapeutic index (ratio of the median lethal dose (LD50) to the median effective dose (ED50)) [2]. Several dozen bioconjugation technologies based on cysteine residues, non-natural amino acids or patterns introduced by molecular engineering have been proposed in preclinical studies [7]. Finally, more tumor specific antigenic targets and optimized release mechanisms of the cytotoxic agent within the tumor have led to the development of more performant ADCs [8],[9],[10].

This review will focus on FDA-approved ADCs, including 3rd generation molecular constructions, and few late stage ADCs among with several strategies for improvement.

2. First- and Second-Generation ADCs approved by the FDA: Design, Mechanism of Action and Therapeutic Indications

The development of immunoconjugates in oncology has enabled the emergence of two key elements necessary to ensure the success of an ADC. The first concerns the need of a linker between the mAb and the payload. This mAb-linker-payload system was first designed with a cleavable linker (Figure 1) assumed to be stable under physiological conditions during plasma circulation, and quickly cleaved after tumor cell endocytosis, in order to selectively deliver the payload in the tumor and limit the appearance of undesirable side effects due to off-target toxicities. This type of linker is sensitive to lysosomal conditions (proteases, acidity and a reducing medium). The second key element for an ADC is correlated with the necessity to have a powerful cytotoxic agent grafted to the antibody. Indeed, the first ADCs were characterized by a low therapeutic index due to the low potency of the payload (e.g., anthracycline), resulting in a very

limited therapeutic effect at the maximum tolerated dose (MTD) of 100 mg/kg. This phenomenon can be explained by the low antigenic density on the cancer cell surface and by the small percentage of ADC able to reach the tumor cell surface compared to the injected quantity (hardly exceeding 0.1%) [11].

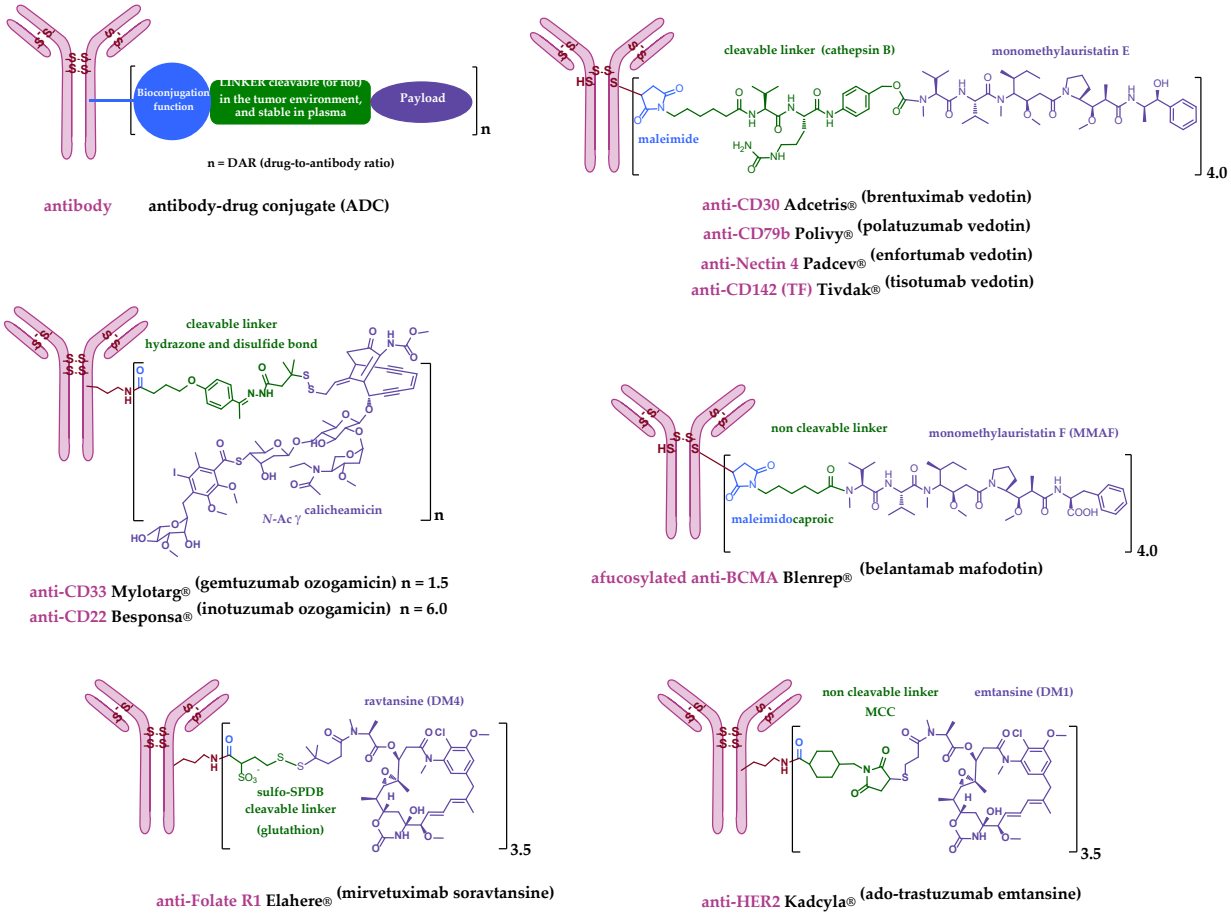


Figure 1: Schematic representation of first and second generation FDA-approved ADCs.

2.1. Mylotarg® and the calicheamycin-based first-generation cleavable linker

Mylotarg® (Gemtuzumab ozogamicin) was approved by the FDA in 2000 for acute myeloid leukemia (AML) [12][13]. Mylotarg® results from the conjugation of calicheamycin, a powerful DNA-cleaving agent [14] with low nanomolar activity, onto gemtuzumab, a mutated anti-CD33 IgG4, via a cleavable linker including an hydrazone bond (Figure 1). This ADC, with an average DAR of 1.5, is a complex mixture with around 50% of unconjugated mAb [15]. After ADC internalization, the hydrazone bond can be hydrolyzed in the endosomal acidic environment to release a precursor of calicheamycin, which then undergoes a reduction by glutathione to the free active calicheamycin. The latter binds to the DNA minor groove and undergoes a Bergman cyclization, which generates a highly reactive diradical causing sequence-selective double-strand cuts. Theoretically, hydrazones should be stable in blood circulation at physiological pH and undergo selective hydrolysis after internalization in more acidic conditions (respectively pH 5.0–6.5 in endosomes and pH 4.5–5.0 in lysosomes). However, the Mylotarg® linker exhibits a certain instability, leading to premature release of calicheamycin in plasma circulation [16], explaining its highly toxic profile and its subsequent voluntary withdrawal by Pfizer in 2010. Mylotarg® benefited from the knowledge accumulated in the clinic in recent years to be reapproved by the FDA in 2017, used at lower doses, with a modified administration schedule and for a different patient population.

A similar linker was developed and used to graft calicheamycin onto inotuzumab, a mutated anti-CD22

IgG4, leading to Besponsa® (inotuzumab ozogamicin, Figure 1), approved by the FDA in 2017 against acute lymphoblastic leukemia (ALL) [17].

2.2. Kadcyla® and the maytansinoid-based second generation linkers

Given these findings, alternative strategies for the design of linkers were necessary to continue the development of ADCs. Thus, Immunogen teams have focused on the use of linkers for the conjugation of maytansine derivatives and incorporating a delivery system using a glutathione-sensitive disulfide bond [18]. These innovative chemically labile linkers were intended to allow controlled release in the presence of glutathione (GSH), the cytoplasmic concentration of which in cancer cells is approximately 1000 times higher than in plasma.

In addition, the careful positioning of two methyl groups neighboring the disulfide bond enabled control of the release kinetics [19]. Thus, the high concentration of reducing molecules in the tumor should have guaranteed a selective release of the payload in the tumor environment but not in the circulation. This type of linker has not yet led to an ADC approved on the market (see Section 5.6).

However, a serendipitous finding allowed Immunogen to identify an unexpectedly effective ADC. The conjugation of DM1 onto the lysine residues of anti-HER2 IgG1 trastuzumab via a non-cleavable heterobifunctional thioether linker containing an N-hydroxysuccinimide ester (succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate or SMCC) led to Kadcyla® (T-DM1 or Ado-trastuzumab emtansine, Figure 1), which was approved by the FDA in 2013 [20]. This new ADC was observed in vitro to be very potent in a HER2-positive breast cancer model, the original structure being active only after internalization and complete enzymatic digestion of the ADC in the lysosome, to obtain the active metabolite lysine-MCC-DM1.

Following this discovery, several observations can be highlighted: (i) the metabolite lysine-MCC-DM1 retains the cytotoxic potential of free DM1, allowing the corresponding ADC to reach an in vitro activity in the picomolar range; (ii) this ADC exhibits no bystander killing effect due to the charged nature of its active metabolite at physiological pH; (iii) an ADC with a non cleavable linker can target only Ag-positive cells and (iv) this ADC has a limited toxicity on normal tissues and is more stable during circulation than an ADC with a cleavable linker.

Immunogen succeeded in developing mirvetuximab soravtansine [21],[22],[23]. This ADC is rather surprising because the release system in reducing conditions (sensitive to glutathione) was too sensitive (because of being unstable in plasma in the early development stage) and the corresponding linker was associated with a lack of success during the development of the ado-trastuzumab emtansine. Mirvetuximab soravtansine therefore results from the bioconjugation of DM4 to an antifolate antibody R1, via a cleavable linker in a reducing medium (rich in glutathione), optimized by the presence of two methyl groups in the alpha of the disulfide bond and by the presence of a sulfonyl group improving the hydrophilicity of the linker and therefore the bioconjugation of DM4. This ADC was approved as Elahere® in November 2022 (Figure 1) for adult patients with folate receptor alpha positive, platinum-resistant epithelial ovarian, fallopian tube, or primary peritoneal cancer, who have received one to three prior systemic treatment regimens.

2.3. Adcetris® and the auristatin-based second generation linkers

In parallel, Seattle Genetics has designed its own linker technology allowing the bioconjugation of dolastatin derivatives (such as monomethyl auristatin E or MMAE) onto the cysteine residues of an anti-CD30 IgG1 to produce Adcetris® (SGN-35 or brentuximab vedotin, Figure 1) [24],[25],[26]. After mild and partial reduction of the interchain disulfide bridges, the anti-CD30 mAb (cAC10) was bioconjugated to a cleavable heterobifunctional maleimide linker. This maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl linker includes a valine-citrulline peptide trigger (ValCit) sensitive to lysosomal cathepsin B and a

para-aminobenzyl alcohol (PAB) as a self-immolative spacer (SIS) allowing the release of MMAE after internalization in CD30-positive tumor cells.

Adcetris® was approved by the FDA in 2011 and for the treatment of anaplastic large cell lymphoma and Hodgkin's lymphoma. After CD30-dependent ADC internalization, followed by degradation of the cleavable linker, the released MMAE can destroy the targeted cell and diffuse across the plasma membrane to reach and kill the neighboring cancer cells. This phenomenon is called the bystander killing effect [27],[28] and allows the released MMAE to kill CD30-positive and CD30-negative tumor cells [29]. By corollary, the bystander killing effect explains the particular in vivo efficacy of Adcetris® in patients treated for heterogeneous lymphomas. Recently, Neri and his team demonstrated that the linker ValCit-PAB used in this ADC can also be cleaved before internalization [30], helping to dispel the dogma stipulating that an ADC must target an internalizing antigen [31] to be relevant (see Section 5.4).

Similarly, this second generation linker (mc-VC-PAB, Figure 1) was used to conjugate MMAE onto polatuzumab, an anti-CD79b IgG1, leading to Polivy® (polatuzumab vedotin, Figure 1), approved by the FDA in June 2019 in combination with bendamustine-based chemotherapy and rituximab, to treat adult patients with diffuse large B-cell lymphoma (DLBCL) [32],[33].

We also found the same linker-drug (Figure 1) in enfortumab vedotin, an ADC resulting from a stochastic bioconjugation on the cysteine residues of an IgG1 targeting Nectin 4, developed by Seattle Genetics, currently in a pivotal phase 2 clinical study [34]. On 16 July 2019, a BLA (Biologics License Application) for this ADC was submitted to the FDA for possible accelerated marketing for patients suffering from metastatic urothelial cancer and previously treated with anti-PD1/PDL1 antibodies. Enfortumab vedotin was finally approved by the FDA as Padcev® in late December 2019.

On the other hand, belantamab mafodotin (GSK2857916), developed by GSK, using the same conjugation technology as the vedotin (maleimide + MMAF) on an afucosylated anti-BCMA IgG1 antibody (Figure 13), has successfully finished a pivotal phase II clinical study against multiple myeloma [35], for patients whose disease has progressed despite prior treatment with an immunomodulatory agent, proteasome inhibitor and anti-CD38 antibody. Following a biologics license application (BLA) filled early in 2020, Blenrep® (Figure 1) has just been approved by the FDA as well as by the EMA, as a first-in-class anti-BCMA therapy against multiple myeloma.

Nevertheless, the molecular architecture of vedotin-based ADC has two limitations. The first is related to the instability of the maleimide used for bioconjugation, which is capable of undergoing a retro-Michael reaction in plasma, causing a partial deconjugation and then a slow transfer to albumin possibly correlated with off-target toxicity [36]. The second concerns the "ValCit" trigger described for Adcetris® as a substrate of carboxylesterase 1C during plasma circulation and of other proteases secreted by neutrophils, which could partly explain the hematological undesirable effects related to Adcetris® (for example, neutropenia) [37],[38],[39].

3. New Strategies in Development for Improved ADC

3.1. Limits of Current Approaches

Many ADCs, in clinical development or in clinical use, are based on a complete internalizing IgG format, targeting an Ag with an extremely high overexpression rate, and are conjugated to tubulin polymerization inhibitors using stochastic bioconjugation techniques [2]. In addition, cleavable linkers are known to be unstable during plasma circulation, while hydrophobic linkers are associated with a higher aggregation propensity. However, ADCs based on a complete IgG are associated with tumor penetration issues in stroma rich tumors [40],[41] and are recycled by the neonatal Fc receptor (FcRn) leading to an undesirable distribution in the endothelium and the liver, responsible for undesirable side effects. Moreover, after

internalization, ADCs effectiveness is based on favorable intracellular trafficking to reach the lysosome, where their degradation will allow controlled drug release. However, this strategy has several limiting factors. Firstly, an ADCs internalization capacity is intimately correlated with the high expression of surface Ag (for example, CD30 and HER2) [42], which explains why these ADCs using conventional tubulin polymerization inhibitors (for example, auristatins and maytansinoids) do not show cytotoxic activity on cells with low antigenic expression. Very powerful drugs (for example, pyrrolobenzodiazepine (PBD) dimers) have been developed to overcome these limitations, but the corresponding ADCs have a limited therapeutic index, particularly in solid tumors. Secondly, internalizing ADCs, including Kadcyla®, induce tumor resistance by several mechanisms. In fact, disturbances concerning internalization, trafficking or recycling of mAb, Ag shedding and defective lysosomal degradation of ADCs lead to a reduced drug release into the cytosol, thus compromising ADCs efficacy [43],[44],[45]. There is therefore today an important need to develop new technologies concerning bioconjugation techniques (leading to homogeneous ADCs), the vector format (antibodies or fragments), the linker (release mechanism) or the drug (new mechanism of action). Each strategy will be discussed as a potential alternative to the heterogeneous internalizing ADCs used until 2019 in clinical practice, to widen the fields of ADCs application [3] and to lead to the recent approval of two third-generation ADCs.

3.2. Site-Specific ADCs

Despite their growing success, until 2019, each approved ADC on the market was in the form of a heterogeneous mixture (variable numbers of cytotoxic drugs on the mAb, in various places), leading to analytical issues [4] during the manufacturing process. Indeed, DAR is not controlled, and this complex mixture significantly influences ADC pharmacokinetics-pharmacodynamics profiles (PK-PD): the naked antibody could be a competitive inhibitor, conjugates with weak DAR have a poor efficacy and those with high DAR are rapidly eliminated in plasma, compromising the ADC therapeutic window. In order to broaden the therapeutic index of ADCs, regiospecific bioconjugation methodologies have been widely developed since 2008 [7],[46],[47]. These site-specific methodologies can be divided into three categories: (i) bioconjugation onto natural or non-natural amino acids [48] [49],[50]; (ii) bioconjugation using enzymes [7],[46],[47],[51] or (iii) linker-based bioconjugation [52],[53],[54],[55],[56].

In a disconcerting manner, despite the development of many technologies allowing a site-specific bioconjugation of a mAb to result in a more homogeneous ADC with an improved therapeutic index (10 in clinical study and more than 40 in preclinical), none of them has yet been validated by the approval of the corresponding site-specific ADC. The only approved homogeneous ADCs are Enhertu® and Trodelvy®, which are not produced through a proprietary site-specific method, but rather result from the conjugation of their respective linker onto the naturally occurring eight interchain cysteines (resulting from the mild and complete reduction of the four interchain disulfide bridges) of their IgG1.

3.3. New ADC Targets

Targeting internalizing antigens on the surface of cancer cells can be extremely difficult in solid tumors rich in intercellular stroma. Thus, a new approach has been developed, consisting of tumor microenvironment (stroma or vasculature) targeting instead of cancer cells [57]. In this strategy, extracellular proteases and other components of the extracellular matrix (such as acidic medium or reducing glutathione) could be used for effective extracellular release of cytotoxics used in non-internalizing ADCs [3].

To this end, Neri and his collaborators have described immunoconjugates derived from a F8 mutant of a SIP targeting the non-internalizing extracellular domain (EDA) of fibronectin. Fibronectin is a component of the tumor subendothelial extracellular matrix, easily accessible to immunoconjugates in comparison to the cancer cell surface [58].

The SIP format results from the fusion of a scFv fragment with the human IgE εCH4 domain [58],[59]. SIP(F8) was also produced with two unpaired cysteine residues at the C-terminal position, allowing regiospecific bioconjugation of two DM1 molecules in order to produce the SIP(F8)-SS-DM1 conjugate with a DAR 2 (Figure 2) [60]. This was compared to its IgG(F8)-SS-DM1 counterpart, carrying an unpaired cysteine residue in the C-terminal position of each light chain (Figure 2). In these immunoconjugates, the disulfide bridge, formed after bioconjugation between the protein and the drug, is sensitive to extracellular tumor-derived glutathione, releasing unmodified DM1 without any residual linkers.

SIP(F8)-SS-DM1 has been shown to be a better candidate than its IgG(F8) -SS-DM1 analogue in therapeutic experiments. The two conjugates were compared in vivo in an F9-teratocarcinoma murine model at equal (equimolar) doses, in a group of five mice, with five injections at 5 mg/kg. The SIP conjugate led to the complete tumor remission in four out of five mice, while only limited tumor remission was observed for the IgG conjugate. The two conjugates have also been shown to present significant differences in terms of release kinetics of the cytotoxic DM1. Neri and his colleagues demonstrated that SIP(F8)-SS-DM1 preferentially accumulates in the subendothelial extracellular tumor matrix and rapidly generates high concentrations of released DM1, contributing to its higher efficiency. This conjugate has also been well tolerated at clinically relevant doses [61]. The advantage of non-internalizing ADCs is the circumvention of certain resistance mechanisms of internalizing immunoconjugates, while the regiospecific bioconjugation method once again allows control of DAR without the presence of DAR 0 species (native unconjugated protein).

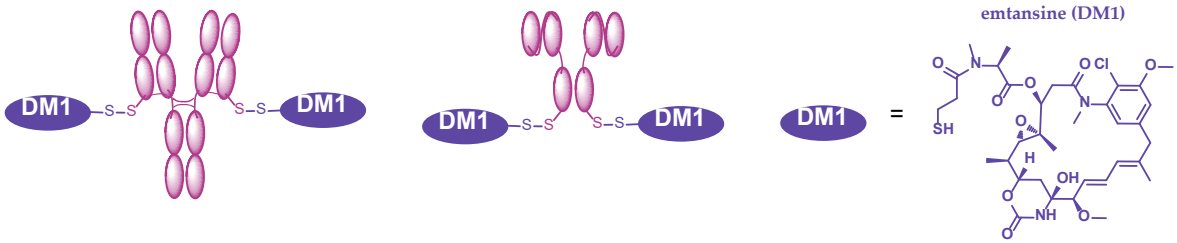


Figure 2: Immunoconjugates IgG(F8)-SS-DM1 and SIP(F8)-SS-DM1 with a DAR 2, and formula of the payload DM1.

3.4. New ADC Release Mechanism

The linker is a key component of ADC, defining its stability and mechanism of action, and particularly the drug release strategy. In a recent study [62], a new cleavable linker was developed, which possesses an Asn-Pro-Val (NPV) sequence sensitive to the human neutrophil elastase (HNE), overexpressed in the tumor microenvironment. This linker permitted the site-specific conjugation of the cell-permeable drug MMAE, onto trastuzumab, using a disulfide re-bridging technology. The resulting ADC TTZ-MC-NPV-MMAE demonstrated retained antigen binding affinity and exhibited high subnanomolar potency against Ag-positive tumor cells after internalization, suggesting an intracellular mechanism of linker cleavage. While no internalization and cytotoxic activity of this ADC was observed on Ag-negative cells in classical conditions, the supplementation of exogenous HNE permitted to restore a nanomolar activity on these cells, suggesting an extracellular mechanism of drug release in these conditions. This in vitro proof of concept tends to prove that the NPV sequence could allow a dual intra- and extracellular mechanism of drug release.

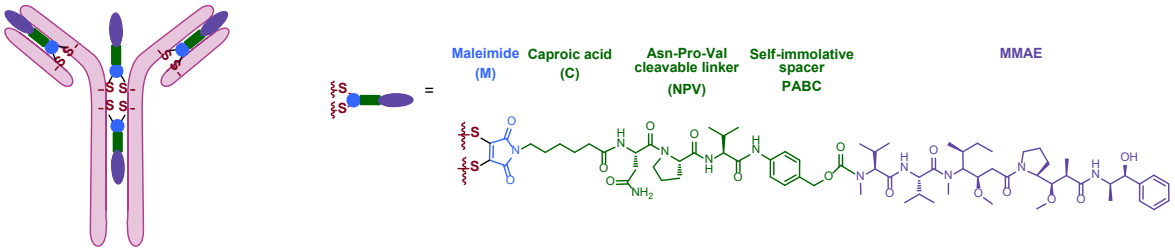


Figure 3: Schematic representation of ADC TTZ-MC-NPV-MMAE and its neutrophil elastase-sensitive linker carrying MMAE.

3.5. Alternative ADC Formats

Despite their efficacy, most ADCs targeting solid tumors have not progressed beyond phase 2 clinical trials, which suggests that there are additional parameters that need to be optimized in order to reach the market [63],[64]. The number of ADCs for solid tumor treatment is limited, and a number of these conjugates have been discontinued due to insufficient activity at repeated doses of DMT. This can be explained by the fact that almost all ADCs are based on a complete immunoglobulin G (IgG) format. Consequently, their effectiveness is limited by their size (150 kDa), associated with sub-optimal penetration and absorption in the tumor [65]. In addition to the size of the IgG, it is now considered that the Fc part of an IgG can be unnecessary or even undesirable for ADCs efficacy [66], although some ADCs (e.g., Kadcyla®) retain the ability of their mAb to exhibit antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP). Indeed, ADCs long half-life induced by the neonatal Fc receptor (FcRn) [67] increases exposure to healthy tissues, while Fc-gamma receptors (FcγR) cross-react with endothelial cells and the immune system, these two phenomena being associated with off-target toxicity [68],[69].

Smaller conjugate formats [66],[70],[71] have been explored to remedy these drawbacks, in particular peptides [72], single domain antibody fragments (sdAb or VHH) [73], single chain variable fragments (scFv) [74], antigen binding fragments (Fab) [75] or small immunoproteins (SIP in the form of scFv dimerized using a CHε4 domain) [60],[61]. As part of this strategy, more recently, the site-specific conjugation of an auristatin derivative to an anti-HER2 scFv (derived from trastuzumab) generated two new scFv-drug conjugates (SDCs, Figure 4) [76]. The intra-tag cysteine (ITC) strategy consisted to incorporate two cysteines near the hexahistidine tag (at the C-terminal position) in order to allow controlled bioconjugation of a heterobifunctional linker comprising of a second generation maleimide, cleavable (for MMAE) or non-cleavable (for MMAF) [3]. The two SDCs retain their affinity for HER2 in comparison to the native scFv and are capable of effectively killing SK-BR-3 HER2-positive cells in vitro at subnanomolar concentrations (EC50 of 0.68 nM and 0.32 nM, respectively). No effect was observed on HER2-negative MCF-7 cells.

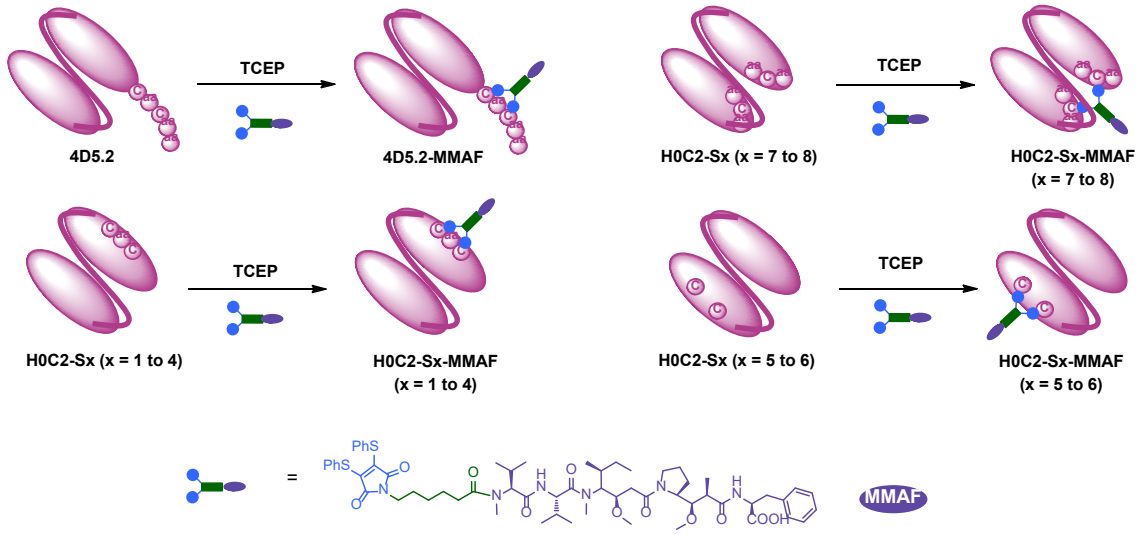
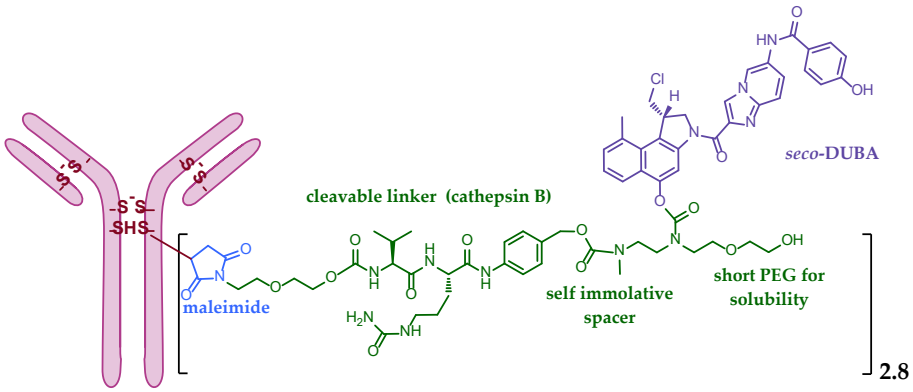


Figure 4: Site-specific SDC (scFv-drug conjugate) with a DAR 1, obtained by mild reduction followed by site-specific bioconjugation reaction, via a dithiophenylmaleimide linker (DSPh) carrying MMAF, onto the disulfide bridge at the C-terminal (intra-tag cysteine strategy or ITC) or in one domain (intra-domain cysteine strategy or IDC) of the scFv.

Nevertheless, innovative solutions are needed to implant cysteines as conjugation sites in the single-chain fragment variable (scFv) format, which is the backbone from which many other antibody formats are built. In addition, the bioconjugation site has the utmost importance to optimize the safety and efficacy of bioconjugates. The previous intra-tag cysteine (ITC) strategy (described above) consisted of introducing a bioconjugation motif at the C-terminal position of the 4D5.2 scFv, but this motif was subjected to proteolysis when the scFv was produced in CHO cells. Considering these data, using three intra-domain cysteine (IDC) strategies, several parameters were studied to assess the impact of different locations of a site-specific bioconjugation motif in the variable domains of an anti-HER2 scFv. In comparison to the ITC strategy, the new IDC strategy allowed to identify new antibody fragment–drug conjugates (FDCs) devoid of proteolysis. FDCs exhibited enhanced stability profiles, better affinity, and better ability to kill selectively HER2-positive SK-BR-3 cells in vitro at picomolar concentrations (best EC50 of 31 pM, similar to the ones of ADC with a DAR of 4) [77].

3.6. New Cytotoxic Agents

As an alternative to the previously described ADCs, Byondis B.V. (formerly Synthon Biopharmaceuticals) developed trastuzumab duocarmazine (SYD985) [78], an ADC with an average DAR of 2.8, resulting from the combination of an anti-HER2 mAb and a duocarmycin precursor (seco-DUBA), linked by a cleavable linker sensitive to cathepsin B, optimized by the presence of two small pegylated units to ensure better solubility (and therefore better conjugation) of the linker-payload moieties (Figure 5). SYD985 is currently being tested against T-DM1 in a phase III clinical study in HER2-positive metastatic breast cancer.



anti-HER2 vic-trastuzumab duocarmazine SYD985

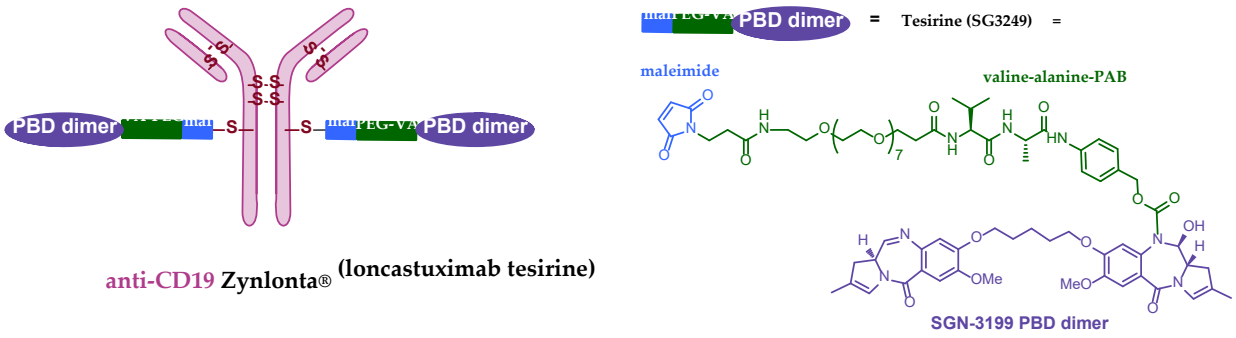
Figure 5: SYD985 formula, anti-HER2 trastuzumab conjugated to seco-DUBA via a cleavable linker sensitive to cathepsin B.

4. Recent approvals and Third-Generation ADCs

4.1. Zynlonta® and the PBD dimer-based ADCs

In parallel, new cytotoxics have been developed to target cancer cells with low Ag expression or resistance to auristatins or maytansinoids.

To this end, PBD dimers have been developed. The molecular structure of these dimers contains two alkylating imine functionalities, capable of forming covalent bonds with DNA. Due to the nature of the DNA adducts and their greater stability, PBD dimers generally have significantly higher antitumor cytotoxicity than PBD monomers. PBD dimers are not substrates of MDR. They are approximately 50–100 times more effective than the conventional cytotoxics used in ADC (MMAE or DM1), exhibiting picomolar activity against many human tumor cell lines (IC50 = 2–7 pM) [79]. PBD dimers were introduced as ADC cytotoxics by Spirogen and Seattle Genetics in the late 2000s. This led to the development of SGN-CD33A (vadastuximab talirine) [49] and SGN-CD70A [50], two ADCs based on PBDs which unfortunately were recently stopped in phase III clinical trials. SGN-CD33A was associated with a higher rate of patient death due to fatal infection and possible liver toxicity, while SGN-CD70A was responsible of thrombocytopenia.



anti-CD19 Zynlonta® (loncastuximab tesirine)

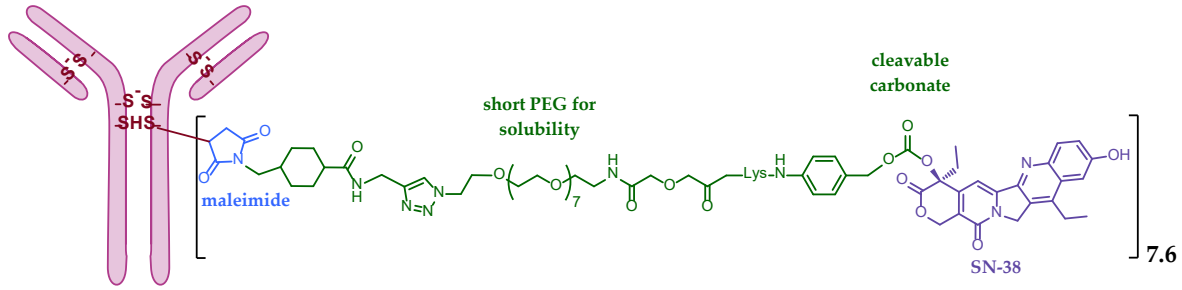
Figure 6: Site-specific ADC Zynlonta® (loncastuximab tesirine) with a DAR 2 carrying tesirine including a PBD dimer (SGN-3199).

Although ADCs using talirine were stopped during clinical trials, two ADCs using the counterpart tesirine are in an advanced phase of clinical study. Rovalpituzumab tesirine (Rova-T or SC16LD6.5) is an anti-DLL3 ADC developed by AbbVie (Stemcentrx) tested in phase III in small cell lung cancer [80],[81]. While ADC

Therapeutics developed loncastuximab tesirine (ADCT-402) [82] and camidanlumab tesirine (ADCT-301) [83], both recently tested in pivotal phase II, respectively against B cell acute lymphoblastic leukemia and Hodgkin lymphoma. Tesirine (SG3249) was designed to combine powerful antitumor activity with desirable physicochemical properties (e.g., favorable hydrophobia and improved bioconjugation). One of the reactive imines is capped with a valine-alanine linker cleavable by cathepsin B. Loncastuximab tesirine (ADCT-402, Figure 6) was approved by the FDA in April 2021 as Zynlonta®, against diffuse large B cell lymphoma (DLBCL) and mantle cell lymphoma.

4.2. Third-Generation SN-38-based ADC Trodelvy®

Among the many companies developing ADCs, Immunomedics designed surprising ADCs by making a triple bet: to build an ADC targeting a slightly overexpressed target, using a system mixing intra- and extracellular release and a less potent payload than those conventionally used in ADC (MMAE and DM1). Sacituzumab govitecan (IMMU-132, Figure 7) is an anti-TROP-2 [84] mAb conjugated to SN-38 (the active metabolite of irinotecan) via a cleavable maleimide linker (acidity) with a short pegylated unit [85]. The FDA-approval of this ADC was delayed and reached in April 2020, after a second BLA (biologics license applications) process was necessary to solve certain CMC issues following a successfully carried out phase III study [86]. Bet successful for Immunomedics: the accomplishment is all the more impressive since this ADC is indicated in refractory or resistant triple negative breast cancer (TNBC) against which there was no treatment until the FDA-approval of Trodelvy® in April 2020 [86]. Another interesting feature of this ADC: the optimization of the linker structure including a pegylated unit led to this ADC with a high DAR of 7.6 [84], without compromising its tolerance or efficiency. DAR 4 has long been considered as optimal, but this statement is now only true for the known approved ADCs with a second generation linker carrying DM1 or MMAE as the payload. IMMU-132 is a very interesting case study demonstrating that the optimal DAR of an ADC will depend on many parameters, mainly the hydrophilic nature of the linker and the grafted payload.



anti-TROP2 **Trodelvy®** **(sacituzumab govitecan or IMMU-132)**

Figure 7: Trodelvy® (sacituzumab govitecan or IMMU-132) formula, ADC with a DAR 7.6, resulting from anti-TROP-2 antibody conjugation to SN-38 via an acid-sensitive cleavable linker.

4.3. Enhertu® and Daiichi Sankyo's Third-Generation DXd-based ADCs

Similarly, in order to conjugate an irinotecan derivative with a meticulously designed linker, the Japanese company Daiichi Sankyo developed DXd (exatecan or DX-8951). DXD is a cytotoxic agent 10-fold more active than SN-38 in vitro on cancer cells. DXD has a better safety profile with an optimized solubility, able to elicit a bystander killing effect [29] to kill neighboring cancer cells, which is an advantage in the heterogeneous tumor, but with a short half-life to avoid off-target toxicity. Bioconjugation of DXd onto the anti-HER2 trastuzumab cysteine residues via a maleimide linker sensitive to proteolysis made it possible to obtain the conjugate fam-trastuzumab deruxtecan-nxki (DS-8201a) with a homogeneous DAR of 7.7 (Figure 8) [87],[88]. Despite its high DAR [89], Daiichi Sankyo's DS-8201a was very well tolerated in rats and

monkeys, and very stable in plasma (2.1% of DXD release after 21 days of incubation, in comparison to the release rate of T-DM1, which was 18.4% after only 4 days, despite the use of a non-cleavable linker). The use of a high DAR compound is remarkable as it contradicts the widely established principle that high DAR conjugates are unlikely to be good candidates due to poor pharmacokinetic profiles. These achievements were possible after testing many linkers. The chosen enzyme-sensitive linker encompasses a tumor-selective GGFC cleavable linker and an amino methylene SIS with a reduced hydrophobicity and a better plasma stability in comparison to the classical PAB SIS. Once DS-8201a is internalized, its linker is selectively cleaved by lysosomal proteases after the GGFG sequence to release a temporary DXD hydrolysate, which SIS amino-methylene is subsequently hydrolyzed to ammonia and formaldehyde to free DXD, triggering cell death.

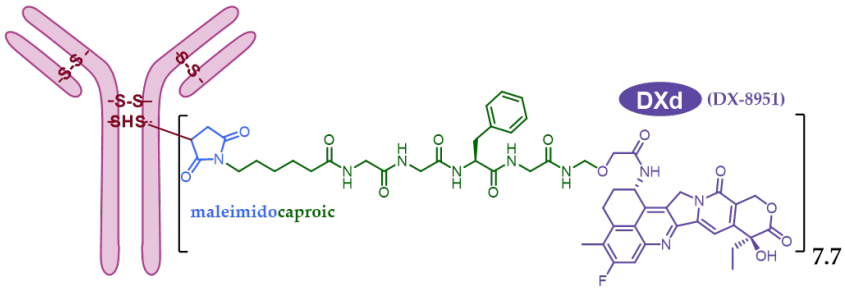


Figure 8: Enhertu® (fam-trastuzumab deruxtecan or DS-8201a) formula, ADC with a DAR 7.7, resulting from anti-HER2 trastuzumab conjugation to exatecan DX-8951 via a linker sensitive to proteolysis.

While Kadcyla® (T-DM1) is known to have in vitro efficacy only against HER2-positive cells with a high HER2 expression level, DS-8201a was effective in the pancreatic Capan-1 cell line with low HER2 expression and in the T-DM1 refractory JIMT-1 HER2-positive breast cancer cell line. With a DAR of 8 and a payload able to elicit the bystander killing effect, DS-8201a can effectively deliver DXD in a heterogeneous tumor in vivo and exhibit a high therapeutic effect [90]. DS-8201a was successfully tested against T-DM1 in a phase III clinical study in metastatic HER2-positive breast cancer last year. Enhertu® was finally approved by the FDA in late December 2019. Based on the promising results of the DESTINY-Breast04 clinical trial, clinicians are starting to differentiate levels of HER2 expression and redefine how metastatic breast cancer is classified with a distinct HER2-low patient population that may be eligible for Enhertu®.

Moreover, ADCs datopotamab deruxtecan (anti-TROP2, ADC DS-1062a) and patritumab deruxtecan (anti-HER3, ADC U3-1402) are both developed by Daiichi Sankyo and currently evaluated in phase 3 clinical trials against triple negative breast cancer. Datopotamab deruxtecan is also tested in phase 3 against non-small cell lung cancer.

5. Quick Overview of ADC beyond Oncology

The success of ADCs as anticancer agents encouraged many researchers and pharmaceutical companies to open the frontier of their application domains beyond oncology. Indeed, recently several ADCs have been developed as immunomodulatory agents or against infectious diseases [91], [92].

5.1. ADC as an Immunomodulatory Agent

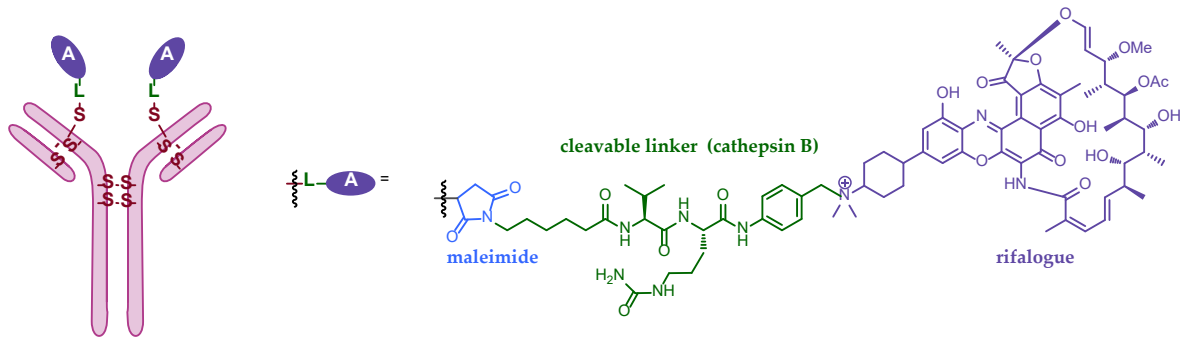
Glucocorticoids (GC) are potent anti-inflammatory drugs but are associated with systemic toxicity leading to serious side effects including but not limited to immunosuppression and metabolic disorders. GC anti-inflammatory effects are very complex but mediated the suppression of tumor-necrosis factor-α (TNF-α) and other cytokines release by macrophages. The most advanced ADC is ABBV-3373, including a GC and

completed a phase 2 in clinical trials, and was developed by Abbvie against rheumatoid arthritis. ABBV-3373 is an ADC resulting from the conjugation of a proprietary glucocorticoid receptor modulator (GRM), derived from dexamethasone, on the anti-TNF-α adalimumab, with a DAR of 2 or 4, according to the bioconjugation process. This latter was optimized using a hydrolyzed maleimide as the bioconjugation moiety, to induce better stability in plasma circulation, by avoiding deconjugation via a retro-Michael reaction. The mechanism of action of ABBV-3373 is a targeted uptake at the surface of immune cells expressing transmembrane TNF, followed by internalization and lysosomal GC release. The released GC then activates the GR pathway and provokes an anti-inflammatory cascade in the nucleus. This targeted mechanism dampens the systemic side effects observed with GC steroids [93].

5.2. Antibody-Antibiotic Conjugate

Staphylococcus aureus is the leading cause of bacterial infections while antibiotic resistance is spreading worldwide. Some S. aureus strains are described to survive standard antibiotic treatment by 'hiding' in host cells like phagocytes, with reduced susceptibility to vancomycin and to resistance to linelozid, daptomycin and all β-lactam antibiotics, with the emergence of methicillin-resistant S. aureus (MRSA).

To circumvent the lack of intracellular bacteria targeting associated with actual treatments, Genentech decided to develop an antibody–antibiotic conjugate (AAC), designed with a carefully selected antibody, linker and antibiotic, to achieve better activity than vancomycin for the treatment of bacteremia in vivo [94],[95]. To achieve this goal, Genentech developed a thiomab directed against pathogen-specific wall-teichoic acids (WTA) present at the bacteria surface. While several antibiotics were tested, only rifalogue, a rifamycin derivative, was selected for its ability to accumulate intracellularly and for its unaltered highly potent bactericidal activity at low pH. This highlights and confirms the fact that, like for ADC in oncology, each AAC component should be carefully selected to achieve the desired therapeutic effect.Rifalogue was site-specifically conjugated onto the anti-WTA Thiomab through a cathepsin B-sensitive linker to produce an AAC with a DAR of 2 (Figure 9). The mechanism of this AAC was its ability to target and fix extracellular S. aureus. S. aureus opsonization leads to its Fc-mediated internalization inside a host phagocyte. This is followed by selective rifalogue release by degradation of the AAC linker in the proteolytic environment of phagolysosome, leading to intracellular bacteria killing. This impressive work could allow some potent antibiotic drugs, forsaken in clinical trials due to host toxicities and unfavorable pharmacokinetic profiles, to be evaluated again as judiciously designed immunoconjugates.



anti-WTA THIOMAB-rifalogue (RG7861)

Figure 9: RG7861 formula, ADC with a DAR 2, resulting from the site-specific conjugation of rifalogue onto an anti-wall-teichoic acid (WTA) Thiomab via a cathepsine B-sensitive linker, targeting the surface of Staphylococcus aureus.

6. Conclusions and Perspectives

ADCs represent today a recent success of an old approach in chemotherapy targeting cancer. The classic internalizing ADCs currently used in clinics are designed to specifically deliver powerful cytotoxic agents to the targeted Ag (+) cancer cells, to eliminate only Ag (+) cancer cells (non-cleavable linker) or all tumor cells including both Ag (+) and Ag (–) cancer cells (cleavable and cytotoxic linker, rather hydrophobic). Over the past decade, ADCs have been improved by the choice of better cytotoxic agents, bioconjugation methodologies, better chosen targeted antigens and optimized antibody engineering.

However, despite their sophisticated design, ADCs are still associated with several limitations (for example, limited solid tumor penetration and toxicity) and the emergence of resistance mechanisms. To overcome these limitations, new antibody formats, new delivery systems, non-internalizing antigenic targets, new cytotoxic agents and site-specific bioconjugation methods have been studied to advance the development of ADCs. Unfortunately, many innovations have not yet been validated for use in clinical protocols, since the resulting ADCs are still in a preclinical or clinical study, and a small number have reached pivotal clinical phase II or phase III.

In conclusion, this field of research offers many encouraging prospects. In the last decade, the search for novel targets and the use of judiciously chosen payloads, with a well-designed release mechanism, have successfully opened the ADC field of applications beyond oncology. Some of these ADCs can fight resistant bacteria. Hopefully, this research may allow forsaken drugs from the past to shine again as new conjugated drugs with better pharmacological profiles and efficacy, and allow ADCs to get a little closer to the magic bullet imagined by Paul Ehrlich at the beginning of the 20th century.

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White Paper

Overcoming the Solubility Challenges of Antibody-Drug Conjugates

Carl Deutsch, PhD

Antibody-Drug conjugates (ADCs) often present solubility challenges due to the hydrophobic properties of the attached payloads; poor solubility can lead to aggregates and impact manufacturability or even pharmacokinetic properties. In an attempt to overcome this hydrophobicity, ADC developers may resort to reducing the drug-antibody ratio (DAR), a strategy which may result in a loss of efficacy, reduce the therapeutic window, and cause unintended side effects. Other opportunities to mitigate solubility challenges are possible and include changes to the formulation, payload, or conjugation site or addition of a co-solvent. Unfortunately, these approaches require additional investments, which can increase development risks as well as have potential intellectual property and freedom-to-operate constraints. If the solubility issue cannot be resolved, the clinical development program may have to be terminated.

Increasing Solubility with ChetoSensar™ Technology

We have developed a chito-oligosaccharide (ChetoSensar™) that dramatically increases the solubility of ADCs when incorporated into the linker-payload construct (Figure 1).

Figure 2 shows the reduction in ADC hydrophobicity when ChetoSensar™ is incorporated into the construct. The top panel illustrates the increase in hydrophobicity of a DAR 4 ADC compared to the unconjugated trastuzumab (Herceptin) monoclonal antibody. When the same DAR 4 ADC has ChetoSensar™ attached, a notable shift towards the unconjugated antibody is observed, indicating that the solubility properties of the ChetoSensar™-ADC are now closer to the unconjugated antibody, facilitating ADC processing in development and manufacturing.

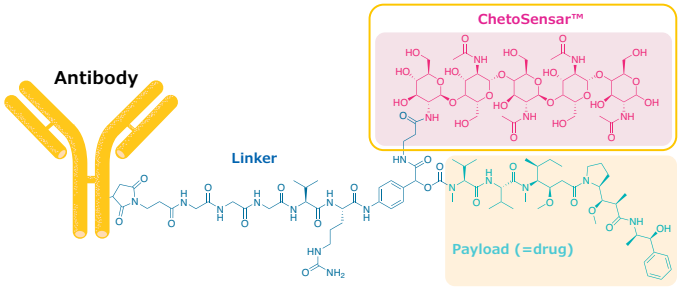

Figure 1. ChetoSensar™ attached to a cathepsin B-sensitive linker and monomethyl auristatin E (MMAE) payload. All ADCs in this report used the monoclonal antibody trastuzumab (Herceptin) as a model.

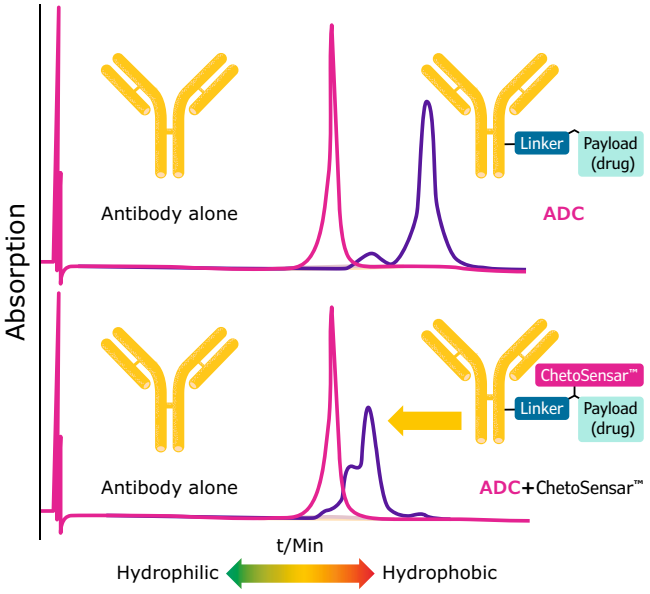

Figure 2. Incorporating ChetoSensar™ into an ADC reduces hydrophobicity. Shown: hydrophobic interaction chromatogram (HIC) with antibody alone (trastuzumab) and DAR 4 ADC with and without ChetoSensar™ (payload is MMAE).

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ChetoSensar™ offers high flexibility when developing ADCs and enables use of a variety of linkers, payloads, antibodies, and conjugation technologies (Table 1).

Linkers	Payloads	Antibodies	Conjugation Technologies
• Disulfide • Cat B • Maleimide • ß-Gluc	• Dolastatins • Maytansines • Duocarmycin • CBI Dimers • PBD Dimers • Camptothecin	• Various IgG formats • Engineered • Bispecific	• Chemical coupling • Enzymatic coupling • Site specific • Stochastic

Table 1. ChetoSensar™ can be used with a wide variety of linkers, payloads, antibodies, and conjugation technologies.

To demonstrate the benefit of ChetoSensar™ in an ADC construct, two ADCs were compared in an *in vivo* study using an SK-OV-3 xenograft (Figure 3). Tumor volume was measured following a single dose of DAR 4 ADC with and without ChetoSensar™. The results showed that at equal doses, the ChetoSensar™ enabled ADC led to rapid and complete tumor regression through day 80, indicating a dramatic increase in efficacy of the ADC.

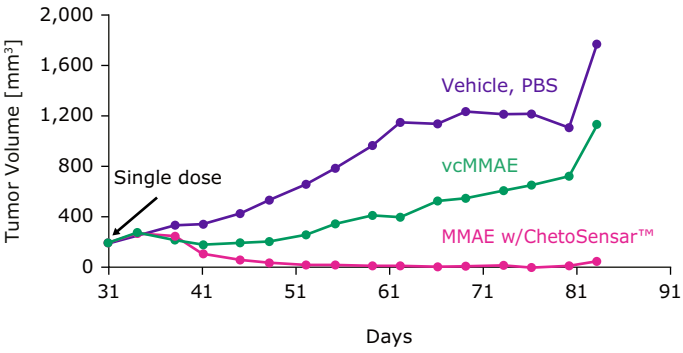
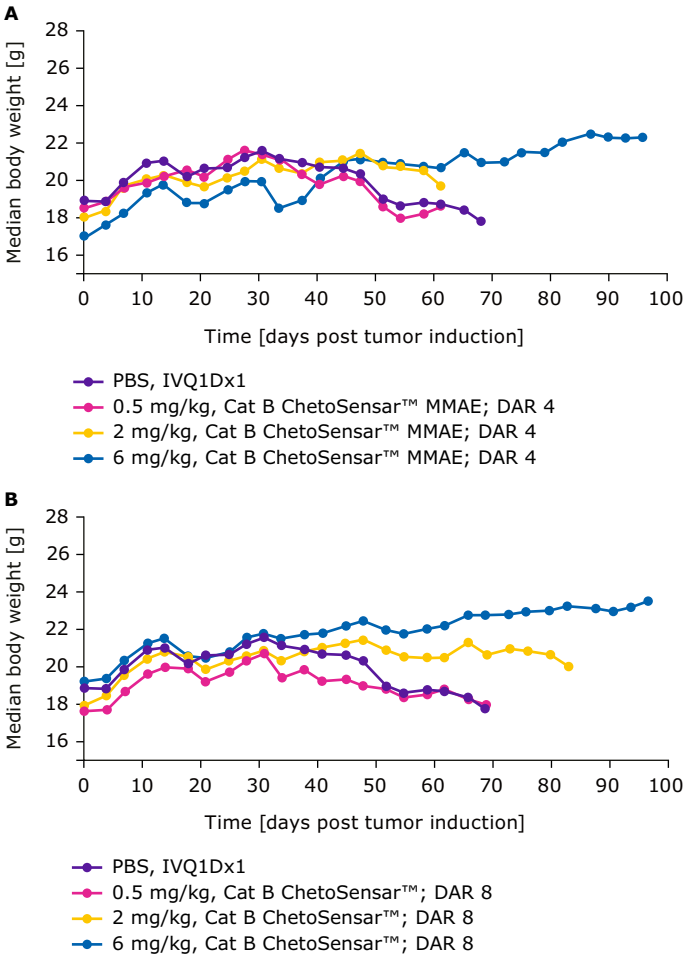


Figure 3. ChetoSensar™-ADC achieves tumor regression close to baseline in SK-OV-3 xenograph. Both ADCs are DAR 4 at 6 mg/kg. Single dose begins at 31 days. All ADCs use traztuzumab as model mAb. Median results report from a group of eight mice.

In addition to efficacy, tolerability of an ADC is critical to its success as a therapeutic. One indicator of tolerability is the body weight of mice throughout the course of an *in vivo* study. With DAR 4 (Figure 4A) and DAR 8 (Figure 4B) ChetoSensar™-enabled ADCs, there was no loss in body weight, indicating the ChetoSensar™-ADCs were well tolerated. In addition, no off-target toxicity of ChetoSensar™ was observed. There were no histological changes in any major organs and no immune response created when added to peripheral blood mononuclear cells (PBMCs) that were isolated from three different human donors (data not shown).

The Basis of Improved Efficacy

We sought to define the reasons for the increased efficacy of ChetoSensar™-enabled ADCs. These ADCs contain the p-aminobenzyloxycarbonyl (PABC) group



- No body weight loss observed
- No side effects observed (e.g. skin rash)

Figure 4. ChetoSensar™-ADCs were well tolerated *in vivo* with repetitive. **A** Body weight of mice in animal study of DAR 4 ADCs with ChetoSensar™. **B** Body weight with DAR 8 ADCs with ChetoSensar™.

that is susceptible to cathepsin B (Cat B) cleavage in the lysosome. PABC linkers have been shown to undergo non-specific clearance and payload loss. However, *in vitro* studies show that ChetoSensar™ may act as a stabilizer against the mouse enzyme carboxyesterase 1C when attached to PABC. The monoclonal antibody Herceptin (trastuzumab) in combination with ChetoSensar™ was shown to be stable in mouse and human cells without any non-specific clearance based on the corresponding carboxylesterase (data not shown). This indicates that in addition to improvements in solubility, the stability of the ADC is driven by improved pharmacokinetics with no negative effect on the binding of the antibody.

We then created a site-specific DAR 2 duocarmycin ADC with ChetoSensar™ and tested it in humanized FcRn mice to predict *in vivo* behavior in humans (Figure 5).

The DAR 2 ChetoSensar™-ADC in buffer solution had a potency in the sub-nanomolar range. *In vivo* plasma samples taken at 4 hours and 168 hours were subsequently tested on antigen-positive cells and showed no loss of efficacy. This indicates that the samples were stable for up to seven days in circulation in humanized FcRn mice. Beyond the DAR 2 studies shown here, ChetoSensar™ empowered the preparation of an aggregate-free DAR 8 duocarmycin ADC, which was previoulsy not possible (data not shown). This reach into higher DAR species with the aid of ChetoSensar™ technology gives optimism to increased potency with other payloads as well.

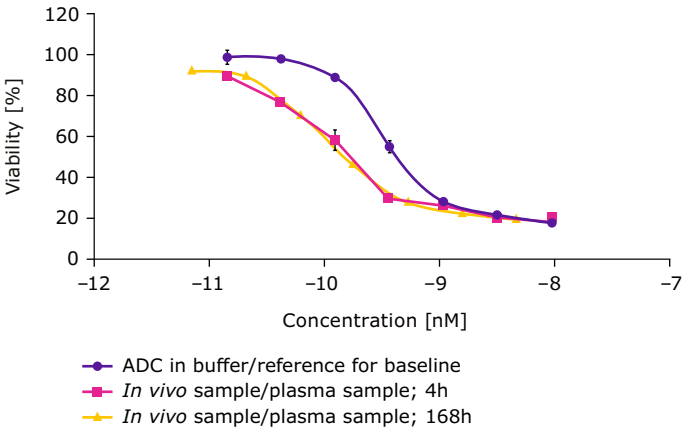


Figure 5. ChetoSensar™-duocarmycin ADC (DAR 2) has favorable pharmacokinetics in humanized FcRn mice. Stability of ChetoSensar™-ADCs is demonstrated with no loss in efficacy after 7 days in antigen-positive cells.

This high degree of efficacy, stability, and robust pharmacokinetics also translated to the *in vivo* setting. As shown in Figure 6, a single dose of DAR 2 ChetoSensar™-duocarmycin ADC led to complete remission of the tumor in a xenograft model with no regrowth through day 100, a notable contrast when compared to the DAR 2 duocarmycin ADC without ChetoSensar™, which had no apparent effect on tumor regression. An ongoing study with a 5 mg/kg dose has also shown complete remission (data not shown).

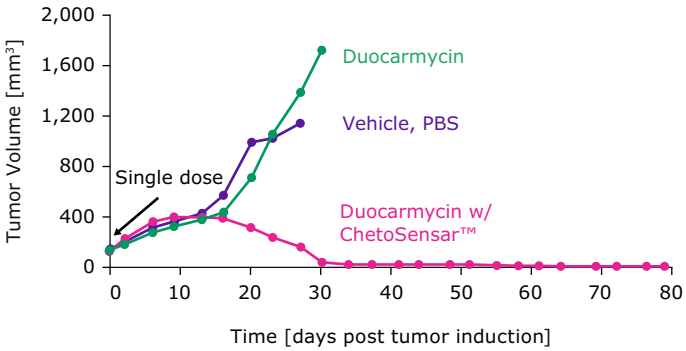


Figure 6. Demonstrated tumor regression with DAR 2 ChetoSensar™-duocarmycin ADC. Purple: Vehicle (PBS); green: ADC without ChetoSensar™; pink: ADC with ChetoSensar™. Both ADCs are dosed at 10 mg/kg; single dose begins at 0 days. ADC construct design referenced in Figure 5.

Importantly, ChetoSensar™ enabled us to make an aggregate-free DAR 8 duocarmycin ADC, which would not have been possible previously.

Solving the ADC Solubility Challenge

The poor solubility of ADCs can put development programs in jeopardy. ChetoSensar™ technology is an innovative option for addressing these solubility challenges. The chito-oligosaccharide linker offers a great deal of flexibility for attaching different kinds of payloads and has been shown to work with a variety of linkers, antibodies, and conjugation approaches. The technology provides access to highly hydrophobic, novel payloads that might not otherwise be considered and presents the possibility of improved efficacy, tolerability, and pharmacokinetics.

The author Carl Deutsch was previously the director for ADC Chemistry and Cell Biology at Merck KGaA.
He is currently the CSO of NBE Therapeutics AG.
For more information about ChetoSensar™, please contact
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02/2023

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- Innovative technologies to advance your drug discovery and development
- Release testing and stability studies for BDS and DP

Do you want to learn more? Contact us:

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Email: giuseppe.camporeale@merckgroup.com

Mickael Guyot
Specialist Merck Life Science Services
Email: mickael.guyot@merckgroup.com

Find out more about our integrated ADC offering on our website:
https://www.sigmaaldrich.com/services/contract-manufacturing/adc-bioconjugation

The Life Science business of Merck operates as MilliporeSigma in the U.S. and Canada.

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UPCOMING MABDESIGN EVENTS

Join us at our next scientific and networking events in 2023

MABDESIGN EVENTS IN S1 2023

PARTNERING EVENT

30 MAY 2023 - TOURS

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THE BIOTHERAPY EXPERT

Information & Registration:
https://mabdesign-partnering-day-2023.mabdesign.b2match.io/

11TH AIS 2023

JUNE 22-23, 2023
TOURS, FRANCE

MAbImprove LabEx Tours Montpellier

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THE BIOTHERAPY EXPERT

Information & Registration: https://aiscongress.com/

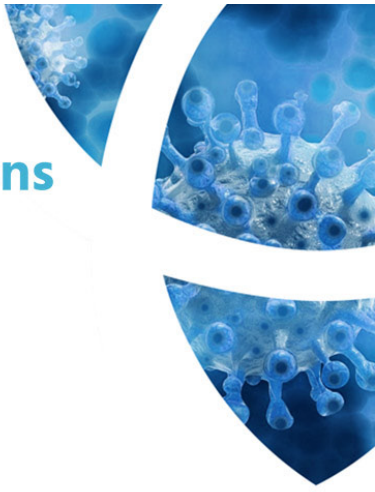
Mabdesign events in S2 2023

8TH BIOPRODUCTION CONGRESS

5-6 October 2023 - Lyon - France

ACCELERATING BIOPHARMA DEVELOPMENT FOR PATIENTS

Information & Registration: www.biopcongress.com



immunotherapies & innovations for infectious Diseases

7th Congress – 22 & 23 November 2023 - Lyon, France

mabdesign THE BIOTHERAPY EXPERT

LYONBIOPOLE AUVERGNE-RHÔNE-ALPES

Information & Registration: www.i4id.org

About MabDesign

MabDesign, the French biotherapy industry association, aims to structure the biopharmaceutical industry in France from its R&D phases to biomanufacturing and marketing. Its objective is also to promote the creation of innovative start-ups resulting from academic research, to increase the visibility of the biopharmaceutical industry, to promote exchanges, to support the development and competitiveness of companies, and to stimulate innovation. Created in 2014 MABDESIGN is administered by ABL Europe, Biomérieux, DBV Technologies, Innate Pharma, Institut Pasteur, Lyonbiopôle, Pierre Fabre, Sanofi, Thermo Fischer, TreeFrog therapeutics and 3 independent field experts.

MABDESIGN OFFERS

STRUCTURE THE INDUSTRIAL SECTOR ...

DIRECTORY | COMMUNICATION | COSSF | EVENT

... to increase its **readability** and international **visibility**.

SUPPORTING THE INDUSTRIAL SECTOR...

CONTINUOUS TRAINING | INNOVATION FINANCING | MARKET STUDY | TECHNOLOGY SURVEY & IP | BUSINESS DEVELOPMENT

... to enhance its **value offer** and **its competitiveness**.

Operational since September 2015, MabDesign currently has more than 260 member companies, including pharmaceutical and biotech companies, service providers, training organizations, high-tech equipment suppliers and specialized consultants.

Next on Watch

- **ImmunoWatch RNA-based drug products**
- **BioprocessWatch Analytical tools**
- **ImmunoWatch Therapeutic Cytokines**

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