Antibody-Drug Conjugates: Targeting the Tumor Microenvironment

Alberto Dal Corso, Samuele Cazzamalli, and Dario Neri

Abstract Antibody-drug conjugates (ADCs) have been used for more than two decades as tools for the selective delivery of cytotoxic agents to the tumor site, with the aim to increase anti-cancer activity and spare normal tissues from undesired toxicity. Until recently, most ADC development activities have focused on the use of monoclonal antibodies, capable of selective binding and internalization into the target tumor cells. However, in principle, it would be conceivable to develop non-internalizing ADC products, which liberate their toxic payload in the extracellular environment. In this Chapter, we review previous work performed on non-internalizing ADC products, with a special emphasis on drug conjugates which selectively localize to the modified extracellular matrix in the neoplastic mass.

Keywords Non-internalizing ADCs · Extracellular tumor antigens · Tumor microenvironment · Vascular targeting

Introduction

Conventional pharmacological approaches for the chemotherapy of cancer are mostly based on the administration of cytotoxic agents, which promote cell death by blocking biological pathways that are essential for cell proliferation. The efficacy of this class of antitumor agents is often limited by their inability to preferentially accumulate at the tumor site, as demonstrated both in preclinical biodistribution studies and in positron-emission tomography (PET) imaging of cancer patients [1]. Drug accumulation in healthy tissues may give rise to side effects, which prevent dose escalation to therapeutically active regimens. For this reason, the covalent

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conjugation of potent cytotoxic payloads to suitable vehicles (e.g., antibodies), capable of binding to tumor-associated antigens (e.g., receptors or other proteins that are overexpressed at the site of disease), has been proposed as a general strategy to improve the therapeutic index of cancer chemotherapy [2].

ADC products result from the conjugation of a cytotoxic agent and a monoclonal antibody (mAb), using a suitable linker. Most of the antibodies that have been used for ADC development display insufficient anti-tumor activity, when administered as "naked" immunoglobulins [3]. On the other hand, the role of the mAb moiety in ADC products mainly consists in the selective delivery of a cytotoxic compound at the tumor site, where the latter is released and acts on cellular targets causing direct damage. According to this mechanism of action, ADCs can be considered as pro-drugs, for which the release of the cargo is of fundamental importance for therapeutic activity.

While ADC products specific to internalizing receptors have shown encouraging clinical responses in patients bearing non-solid tumors, the therapeutic activity against the most frequent solid malignancies (e.g., tumors of breast, lung and colon) is still far from optimal [4]. The emerging clinical results are often less favorable than the preclinical data obtained in tumor-bearing mice, where several internalizing ADC products have led to cancer cures. The higher permeability of interstitial tissues in mice xenografts, compared to solid malignancies in human patients, may partially account for this observed discrepancy [5]. A suboptimal penetration of ADC products within the tumor mass may result in an insufficient delivery of suitable payload concentrations. The limited diffusion properties of monoclonal antibodies emerged from immunofluorescence detection studies, which revealed the striking accumulation of mAbs in IgG formats on perivascular tumor cells, with a substantial inability to penetrate the tumor mass and to reach the majority of neoplastic cells [6].

Since the birth of the ADC technology, it has commonly been assumed that the mAb should preferably be directed against tumor-associated antigens expressed on the surface of cancer cells. Ideally, the ADC would internalize upon binding to its cognate target, thus facilitating the delivery and release of the cytotoxic cargo inside the malignant cell. This receptor-mediated endocytosis represents the most exploited mechanism for ADC activation, as discussed extensively in different reviews [7].

In principle, it is conceivable that also ADC products, based on internalizing antibodies, may display at least part of their activity through drug release in the extracellular space. The internalization efficiency is typically variable and rarely reaches 100%. Furthermore, while antibody internalization can be easily studied *in vitro*, an *in vivo* characterization of the process is hindered by many technical limitations, associated with the processing of the tumor mass and with the specific detection of individual antibody, linker and payload components. As a result, the need to use internalizing mAbs for ADC development has recently been questioned and the availability of novel antibodies, with exquisite tumor-targeting properties, has prompted the investigation of ADC products based on non-internalizing ligands.

In the following sections, the development and *in vivo* testing of non-internalizing ADC products is described, with a special focus on molecules targeting the modified extracellular matrix within tumor lesions.

Non-internalizing ADC Products: Mechanism of Action

As an alternative to the traditional receptor-mediated endocytic process, the drug release from tumor-targeting devices could ideally take place in the tumor microenvironment, allowing the subsequent diffusion of the active payload and its internalization into neighboring neoplastic cells. Since passive diffusion is a non-specific process, the cytotoxic agent has the potential to reach antigen-negative cancerous cells within the tumor mass (a mechanism often referred to as "bystander effect"). In principle, non-internalizing ADCs may display potent therapeutic activity against tumors with high mutation rates or characterized by antigen loss, where certain cell populations can develop resistance to conventional internalizing ADCs. Potentially, the bystander killing effect could also impair structures which support tumor growth, such as stromal cells, leukocytes and tumor blood vessels, thus enhancing the anti-tumor effect of the product [8].

Ideally, this alternative strategy could be potentially pursued using monoclonal antibodies specific to both tumor-specific extracellular structures and poorly/non-internalizing transmembrane antigens. However, due to the identical localization of the target protein, mAbs targeting non-internalizing transmembrane receptors would show similar features in terms of tumor accumulation, as compared to antibodies specific to internalizing antigens. Together with the mAb development and the choice of a suitable payload, the design of a proper linker is crucially important for the generation of efficacious and well-tolerated ADCs. While both cleavable and non-cleavable linkers have found application in internalizing ADC products, only cleavable bonds have been so far used as linkers of choice, for the development of non-internalizing drug delivery systems. This can be easily explained by the intrinsic nature of the endocytic process, which leads to the proteolytic degradation of the antibody structure in the intracellular compartments, followed by the release of an active drug metabolite. Various cleavable linkers have been proposed for the preferential drug release in the tumor interstitium. A main requirement to prevent premature drug release and the related side effects is a high linker stability in plasma, after ADC administration. Provided that a sufficient amount of the ADC reaches intact the tumor microenvironment, a second key attribute of the linker is the ability to efficiently release the payload at the tumor site. Glutathione (GSH) represents the most abundant thiol and reducing agent in the intracellular space, both in normal cells and in tumors, which often contain higher concentrations of this species [9, 10]. While disulfide-based linkers have been designed for the intracellular release of anti-cancer drugs, the same chemical structures can be considered for the extracellular drug release, as a consequence of tumor cell death and increased GSH concentration. Disulfides are typically stable in the absence of free thiols at physiological pH, with a serum half-life that can be longer than 1 week. *In vivo*, certain disulfide-based ADCs have exhibited stability in blood for 2–4 days. Moreover, this stability can be dramatically improved by increasing the steric hindrance of substituents at the cleavage site [11]. Several ADCs and small molecule-targeted cytotoxics that incorporate reducible linker systems such as disulfide bridges have been considered

for clinical development [2, 12]. Most of these new products have been designed to release the payload through receptor-mediated endocytosis. However, it is conceivable to assume that the tumor environment *in vivo* is a more complex scenario, in which dying cells are constantly releasing reducing agents to the surrounding areas. Therefore, non-internalizing ADC products based on disulfide bonds can potentially be cleaved in the tumor extracellular milieu, releasing the payload and promote apoptosis in cancer cells. The release of GSH to the extracellular environment may generate a self-amplifying cycle of cell death and subsequent drug release (Fig. 1).

In addition to disulfides, certain peptide sequences have been used as linkers for the generation of ADC products. These functional groups combine a high systemic stability with a rapid release of the drug at the site of disease. Indeed, proteolytic enzymes such as cathepsin B, urokinase-type plasminogen activator and matrix metalloproteinases (MMPs), which are involved in cancer progression features like angiogenesis, invasion and metastasis [13], may be over-expressed at the tumor site, both in intra- and extra-cellular compartments [14]. In particular, the Valine-Citrulline (Val-Cit) dipeptide had shown promising features for the development of internalizing ADCs. This line of research led to the use of a Val-Cit-containing linker in the marketed Adcetris™ product and in other clinical-stage candidates [15, 16]. Similarly to the cleavage of disulfide bonds, proteolytically-cleavable linkers could be exploited also for the release of drugs in the extracellular tumor microenvironment. Indeed, the protease-mediated release of payloads from non-internalizing ADCs can be amplified by tumor cell death, which sheds a large number of proteolytic activities into the cancer microenvironment (Fig. 1).

Non-internalizing ADC Products: Early Evidence of Biological Activity

Studies on non-internalizing (or poorly internalizing) ADC products have been performed both against targets expressed on the cell membrane (such as CD20, CD21, CAIX and FAP) and against components of the modified extracellular matrix in the neoplastic mass (e.g., splice variants of fibronectin and tenascin-C, fibrin and collagen IV). CD20 and CD21 are well-known cell-surface markers of B-cell derived non-Hodgkin's Lymphomas (NHLs) and have been intensively investigated as antigens for ADC products [17, 18]. NHLs have been extensively studied as targets for ADC development, providing insights into the mechanism of action, the anti-tumor potential and limiting toxicities. NHLs are often successfully treated with a combination of chemotherapeutic agents and antibody-based products [19]. However, there is a need for improved medications, especially for patients who relapse from previous pharmacological interventions. CD19 and CD22 have been described as internalizing NHL antigens, while anti-CD20 and anti-CD21 antibodies typically remain on the membrane of B cells and lymphoma cells [20]. Polson and coworkers generated ADCs against different NHL antigens (i.e. CD19, CD20, CD21, CD22, CD72, CD79b, and
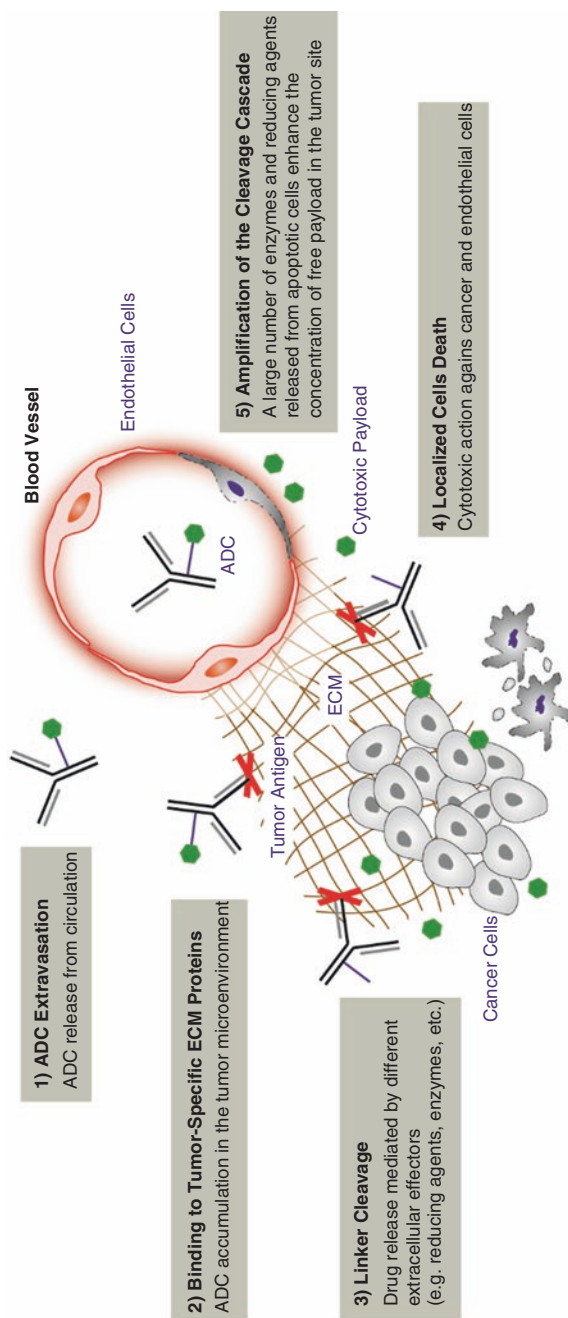
Fig. 1 Schematic representation of possible mechanisms for the drug release and cytotoxic activity of non-internalizing ADCs

CD180), in which potent anti-tubulin agents (DM1 or MMAE) were conjugated to the parental antibodies through either cleavable (disulfide or peptide bonds) or non-cleavable linkers [17]. Therapy experiments performed in tumor-bearing mice showed that all ADCs featuring cleavable linkers (i.e., both products based on internalizing and on non-internalizing antibodies) exhibited a therapeutic effect *in vivo*. By contrast, when non-cleavable linkers were used, only the products directed against internalizing antigens showed a therapeutic activity. Similar results were reported for an anti-CD20 antibody coupled to calicheamicin through both cleavable and non-cleavable linkers [21]. These observations are compatible with the assumption that the cleavable linker is processed in the tumor extracellular space after ADC localization. Subsequently, the drug may diffuse through the cell membrane, reaching its biochemical target. This mechanism of action was reinforced by the observation that the substitution of MMAE with its charged analogue MMAF (i.e., membrane-impermeable) led to a lower antitumor activity, for ADC products directed against non-internalizing antigens [22].

Investigated as tumor marker for the development of targeted cytotoxics, Carbonic Anhydrase IX (CAIX) has long been considered to be an internalizing antigen. However, recent studies have clearly shown that CAIX displays extremely poor internalization properties and resides virtually exclusively on the cell membrane. Carbonic Anhydrases are metalloenzymes that can be found in most of living organisms, where they catalyze the hydration of carbon dioxide to bicarbonate. Among all the CA isoforms, CAIX (formerly referred to as MN antigen) is a trans-membrane homodimeric enzyme overexpressed in more than 90% of clear cell renal cell carcinoma (ccRCC) subtypes [23]. In addition, CAIX is one of the best markers of hypoxia and, as such, can be found in many tumor types, especially those characterized by a low oxygen concentration [24]. The pattern of expression of CAIX in healthy organs is limited on the first portion of the gastro-intestinal tract (e.g., stomach, duodenum and gallbladder) [25], These encouraging immunohistochemical results stimulated the investigation of CAIX as a target for ADC products. Although in the early development anti-CAIX therapeutics were designed to be internalized by tumor cells, our group recently reported the inefficient internalization of the protein upon ligand binding [26, 27]. Petrul and coworkers explored the conjugation of an anti-CAIX mAb to MMAE, through the cleavable Val-Cit linker, to generate the ADC BAY 79–4620 [28]. The group demonstrated the selective affinity of this product to the CAIX isoform and its ability selectively kill CAIX-positive cancer cells *in vitro*, by tubulin disruption. BAY 79–4620 was also shown to be effective *in vivo* in mice grafted with HT-29 and Colo205 colorectal tumors or with cervix carcinoma HeLa-MaTu tumors, at doses between 5 and 10 mg/kg. A modest anticancer activity was reported against other cancer models, albeit at higher doses (30–60 mg/kg). However, while free MMAE (0.2 mg/kg, equivalent to 10 mg/kg of ADC) was less effective than BAY 79–4620, the efficacy of paclitaxel administered at the dose of 15 mg/kg was comparable to the one described for the ADC. In 2014, BAY 79–4620 entered a phase I dose-escalation clinical study with 12 patients, bearing histologically or cytologically confirmed solid tumors [29]. The product was administered at doses ranging from 0.3 to 4.6 mg/kg. While no complete or partial response

were reported, treatment-related side effect occurred in the majority of patients and the highest dose led to patient death due to cardiac arrest and pancreatitis. This tragic event underlined the importance of an accurate preliminary evaluation of the antigen expression in patients, since data of CAIX expression in the studied tumors were available for only 50% of patients, among whom only 2 showed more than 10% antigen-positive cells in the tumor mass.

Antigens that localize in the tumor microenvironment, or on the surface of stromal cells have also been studied as targets for non-internalizing ADCs. The extravascular deposition of fibrin has been described in different human solid tumors as a consequence of the disruption of vascular barriers, which allows the extravasation of fibrinogen and other substrates of the coagulation cascade [30]. Indeed, after tumor transplantation in animal models of cancer, fibrin deposition is one of the first morphological changes that can be observed [31]. While in wound healing processes fibrin is progressively replaced by collagen fibers in few weeks, fibrin clot formation persists in cancer until living tumor cells are present [32]. Yasunaga and coworkers exploited this tumor-specific pathophysiological feature to develop the first fibrin-specific ADC [33]. This immunotoxin comprised a chimeric IgG1 mAb coupled to the active metabolite of Irinotecan (SN-38) as payload. Cysteine residues of the immunoglobulin were coupled to dendrimeric structures bearing 3 SN-38 molecules, individually bound to a PEG spacer via ester linkers. Such a complex design allowed a heavy functionalization of the mAb scaffold, achieving a drug/antibody ratio (DAR) of approximately 24. The resulting anti-fibrin immunoconjugate was stable at acidic pH values, but released gradually and effectively SN-38 at physiological pH in saline buffer and in mouse serum. As expected, this so-called AFCA-branched-PEG-(SN-38)$_3$ ADC acted as a pro-drug *in vitro*, displaying no substantial direct activity against tumor cells. By contrast, four injections per week of the product into tumor-bearing mice at a dose of 13.3 mg/kg were able to suppress tumor growth for more than 1 month. This potent anti-tumor activity was remarkable, especially when compared to the administration of Irinotecan (injected daily at the MTD) which was largely inefficacious. A long-term observation of side-effects revealed that the ADC product was well tolerated in mice, with no signs of bone marrow, liver or kidney dysfunction at the recommended dose. Immunohistochemistry and *in vivo* fluorescence endomicroscopy indicated that the antitumoral activity was mainly due to tumor vessel disruption.

The same group working on anti-fibrin ADCs also reported activity for products directed against murine collagen IV [34]. The naked antibody was coupled to eight molar equivalents of SN-38 cytotoxic agent via a PEG spacer and a cleavable ester linker. The group compared the anti-collagen-4 conjugate and another ADC product, targeting the EpCAM, an antigen expressed on the cancer cell membrane. The comparative evaluation of the two products highlighted the potential of ADCs targeting the tumor stroma to localize and efficiently release their toxic cargo within the tumor microenvironment. Indeed, some cell-targeting products are hindered by stromal barriers, preventing access to the antigen on the cell membrane. When comparing the two products, the anti-collagen IV ADC was found to be superior in two different EpCAM-positive murine models of carcinoma. Since the treatment with

the collagen-targeted ADC resulted in a higher SN-38 concentration in the tumor and with the death of vascular endothelial cells, the authors concluded that the uneven distribution of the anti-EpCAM product within the tumor mass may have led to an inferior performance and lower efficacy.

The work by Yasunaga and colleagues highlighted the potential of stromal-targeting ADCs. Metastasis and tumor invasion are usually linked to adaptation of mesenchyme-derived stromal cells (fibroblasts, myofibroblasts, endothelial cells, pericytes, smooth muscle, and hematopoietic cells) of the neighboring healthy organs [35, 36]. Fibroblasts respond to cancer progression producing Fibroblast Activation Protein α (FAPα), a serine protease involved in tissue remodeling and wound healing [37]. This antigen has been initially proposed as a possible target for cancer therapy with unconjugated antibodies in colorectal cancer patients. Sibrotuzumab, a humanized anti-FAPα antibody, was found to be well tolerated and to exhibit a selective tumor uptake 24–48 h after i.v. administration. No anticancer activity, however, was detected in patients [38]. Ostermann and colleagues conjugated an anti-FAPα mAb to different maytansinoid payloads using both cleavable and non-cleavable linkers [39]. The internalizing behavior of the FAPα antigen was demonstrated by cell antiproliferative assays *in vitro*, where all the ADC products, including the ones featuring non-cleavable linkers, were found to be active against FAPα-transfected cells. However, the *in vivo* administration of the ADCs in mice bearing a panel of FAPα-positive tumors (i.e., pancreatic, a non-small cell lung, a head and neck squamous cell and a colorectal carcinoma) revealed that cleavable linkers are required to induce a potent anticancer effect. These data suggested that the efficacy of the anti-FAP ADC products was due to a bystander effect, associated with the diffusion of the active payload within the tumor microenvironment. In line with this proposed mechanism of action, histological analysis and biomarker studies identified the death of malignant cells surrounding stromal cells as an early therapeutic event.

Another example of a tumor-associated antigen expressed on fibroblasts in the tumor stromal environment is LRRC15 (i.e., leucine rich repeat containing 15). Also known as Lib, this protein is a transmembrane member of the leucine-rich repeat superfamily, which are involved in cell-cell and cell-ECM interactions. Showing weak expression in healthy tissues, LLRC15 was initially detected in astrocytes in response to pro-inflammatory cytokines [40]. It was then found to be frequently overexpressed in many solid tumors, such as aggressive breast cancer [41] and prostate tumors [42]. The anti-LRRC15 ADC product ABBV-085, based on the linker-toxin combination ValCit-MMAE, has recently entered Phase I clinical trial, after showing promising activity against different murine tumor models, administered both as single agent and in combination with chemotherapy, radiation or checkpoint inhibitors [43].

In general, it is technically challenging to quantify internalization rates *in vivo*, even for products directed against cell surface targets. While the examples reviewed below relate to non-internalizing ADC targets (e.g., extracellular matrix antigens), it is reasonable to assume that a substantial portion of putative internalizing ADCs may not reach the corresponding intracellular compartments *in vivo* as intact conjugates.

Targeting the Modified Extracellular Matrix in Tumors

Among the clinically-validated tumor markers, specific isoforms of ECM proteins represent ideal targets for biopharmaceutical intervention. The generation of these tumor-specific proteins can be considered as an end-product of the abnormal proliferative rates of cancer cells, which is not only sustained by several defections in fundamental inhibitory functions of neoplastic cells (e.g. contact inhibition, apoptosis, autophagy, cellular homeostasis) but it is also favored by substantial alterations of the extracellular environment [44]. In particular, the high proliferation rate and the irregular vascularization of a fast-growing tumor mass lead to inadequate oxygen supply to the tumor tissue. It is now well established that cancer cells modify their metabolism to adapt to hypoxia: cellular respiration runs under anaerobic conditions, causing high glucose consumption and production of large quantities of respiratory end-products (i.e. CO_2 and H^+-lactate) [45]. The latter are released in the extracellular environment, resulting in a substantial acidification of the tumor interstitium (the pH can shift from the usual values of 6.5–7.0 to values as low as 6.0). While this phenomenon may lead to apoptosis in normal cells, it acts as a Darwinian selection process for cancer cells, which eventually develop resistance to the altered environmental conditions. For instance, the enzymes carbonic anhydrase IX and XII are over-expressed in many tumors to catalyze the CO_2 hydration in the extracellular environment. This process minimizes the passive diffusion of CO_2 through the membrane, thus allowing the cell to maintain a slightly alkaline intracellular pH (pH 7.2–7.4), which results from increased metabolism and supports cell proliferation [46].

Anomalies in pH values at both side of the cell membrane have been associated with the expression of proteins in mutated isoforms, generated by alternative splicing of their primary RNA transcript. Although the latter is a fundamental process in many physiological functions (e.g. in tissue and organ development) [47], the understanding of alternative splicing in cancer is a field of growing interest in oncology, to such an extent that aberrant alternative splicing is now commonly included in the list of the hallmarks of cancer [48, 49]. Alternative splicing events can generate protein isoforms that help tumors to acquire therapeutic resistance. Moreover, protein splice variants have been associated with particular diagnostic and prognostic features for certain types of cancers, even though their functional/mechanistic role is often not understood [50]. For instance, acidification of the tumor microenvironment have been shown to influence the alternative splicing of vascular endothelial growth factor (VEGF-A) in by endometrial cancer cells [51]. The produced isoforms are known to activate signaling pathways that stimulate tumor progression (e.g. angiogenesis and metastasis) and thus represent a mechanism of tumor cells adaptation to the acidic stress.

A basic intracellular pH may lead to the modulation of splice variants for certain extracellular matrix (ECM) components, such as fibronectin and tenascin C [52, 53]. Fibronectins (FNs) are glycoproteins, which are present either in soluble form in plasma and other body fluids or in cellular form in the ECM and basement membranes of tissues. Acting as a bridge between the cell surface and the extracellular

Oncofetal Fibronectin

Oncofetal Tenascin C
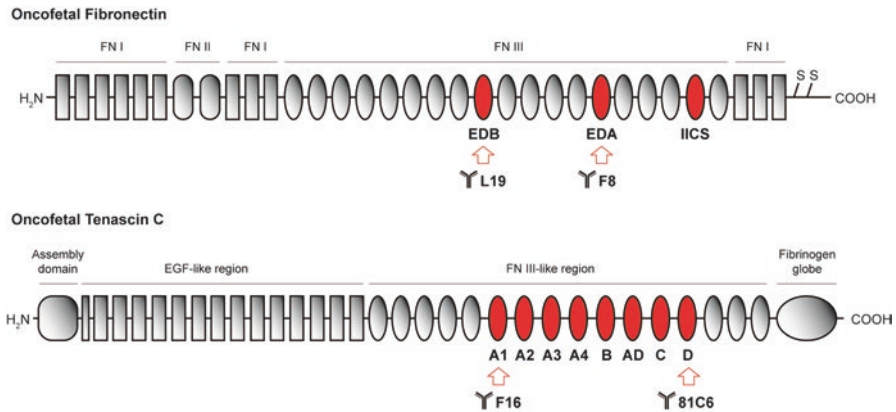
Fig. 2 Modular structure of oncofetal variants of ECM proteins fibronectin and tenascin C. Different shapes are given to different types of protein repeats. Alternatively-spiced domains are shown in red. Arrows show clinically-evaluated domains and their relative antibodies

material, FN is involved in various cell-ECM interactions, such as adhesion, cell migration, hemostasis, thrombosis and wound healing [54]. FN is secreted from cells as a dimer consisting of two 250 kDa subunits covalently linked by two disulfide bonds near their C-termini. Although FN is encoded by a single gene, it exists in multiple isoforms which result from the combination of three alternatively-spliced domains: EDA, EDB and IIICS (see Fig. 2) [55]. While EDA and EDB show constant structures, composed respectively by 90 and 91 amino acids, the extra domain IIICS can be expressed in multiple variants in humans, ranging from 64 to 120 amino acids [56]. The group of Luciano Zardi firstly reported the over-expression of FN extra-domains in tumor-derived or SM40-transformed human cells, compared to normal human fibroblasts [57].

This discovery stimulated an intense research activity around FN splice variants, aimed at understanding their expression pattern and pathological role. In particular, the EDB of FN was found to be virtually absent in all normal adult tissues, but abundantly expressed in the proximity of angiogenic blood vessels and in the stroma of various types of aggressive tumors, including brain, lung, skin, kidney and bladder [58, 59]. Similarly to EDB, the EDA domain was found to be expressed in subendothelial ECM of proliferating tumors, while being undetectable in human plasma and healthy tissues [60]. The singular expression profile of EDA and EDB led to the identification of these markers of angiogenesis as "oncofetal" domains of fibronectin [61, 62].

Tenascin C (TnC) is another cell-binding, large oligomeric glycoprotein of the ECM, composed by 240 kDa subunits that assembly in oligomers (mainly hexamers) through disulfide bonds [63]. A functional antagonism between TnC and FN have emerged from different observations: (i) TnC shows poor binding affinity of to ECM components (FN, collagen, laminin), thus supporting only a weak cell attachment to ECM; (ii) TnC promotes cell rounding and detachment, whereas FN pro-

motes cell-substrate adhesion; (iii) FN is ubiquitously distributed while TnC expression is restricted to morphogenesis and remodeling events [64]. Two main human TnC isoforms are generated by alternative splicing of the single TnC primary mRNA, resulting in the inclusion (or omission) of eight extra domains (Fig. 2) in the final transcript. The expression of these two isoforms was proposed to be dependent to intracellular pH, as a result of adaptation to environmental conditions. In particular, while TnC alternative splicing in normal cultured fibroblasts showed a sensitivity towards small variation of extracellular pH [65], malignantly-transformed cells mainly expressed the large TnC variant (i.e., bearing the 8 extra domains). This observation was explained by the ability of malignant cells to maintain a basic intracellular pH even in an acidic environment, which promotes the alternative splicing event [53].

The abundant and tumor-specific expression of oncofetal FN and TnC stimulated the investigation of these proteins as ideal targets for biomolecular intervention. For instance, ^{131}I-labeled murine and chimeric antibodies specific to A1 and D domains of TnC have been evaluated in the clinic for the treatment of glioma and lymphoma [66, 67]. Moreover, the human recombinant antibodies L19 and F16 were generated upon selections of a phage display library against the EDB and A1 antigens [68, 69]. The two antibodies have been produced in different formats (scFv, diabody, SIP, IgG) and their tumor-targeting properties were studied by quantitative biodistribution analysis, revealing promising *in vivo* tumor targeting performances [69, 70]. Importantly, quantitative biodistribution data are available for L19 and F16 both in mice and in man. The ^{131}I-L19 and ^{131}I-F16 antibodies in SIP format have been evaluated for radio-immunotherapy applications in patients bearing Hodgkin's lymphoma [71, 72] and head and neck cancer [73]. In addition to radiopharmaceutical applications, a variety of immunocytokines composed by the L19 and F16 antibodies fused with either interleukin 2 or TNF are currently evaluated in the clinic for the treatment of different solid tumors (i.e. melanoma, soft tissue sarcoma, diffuse large B-cell lymphoma, oligometastatic solid tumor, Merkel cell carcinoma, acute myeloid leukemia and non-small cell lung cancer) in combination with chemotherapy. Similarly to L19 and F16, also the F8 antibody (specific to the EDA domain of FN) may be considered as a delivery vehicle for pharmaceutical applications. F8 displayed encouraging tumor-targeting properties in mouse models and a characteristic ability to stain neo-vascular structures not only in aggressive solid tumors, but also of solid masses of hematological malignancies [74]. EDA is expressed not only in cancer, but also in other pathological conditions, characterized by extensive tissue remodeling. The observation of an intense and diffuse staining pattern of F8 in synovial tissue biopsies obtained from rheumatoid arthritis patients led to the development of the immunocytokine F8-IL10, which is currently evaluated in the clinic [75].

The L19, F8 and F16 antibodies, specific to non-internalizing ECM antigens, have been instrumental for the selective delivery of cytotoxic compounds to the tumor environment. Initial studies involved the functionalization of the anti-EDA F8 antibody with cemadotin, a tubulin inhibitor [76]. A thiol derivative of this dolastatin analogue, with low-nanomolar cytotoxic activity, was coupled in a site-

specific manner to two C-terminal Cys residues of the F8 antibody in SIP format. The resulting ADC showed a drug-antibody ratio (DAR) of 2 and the disulfide linker displayed acceptable stability in mouse plasma (half-life of approximately 48 h). On the other hand, the ADC incubation with glutathione resulted in a fast and "traceless" release of the drug in its active thiol form. Therapy experiments performed in immunocompetent mice, subcutaneously grafted with F9 teratocarcinoma cells, showed a substantial tumor growth inhibition. Most probably, the disulfide linker can be cleaved by glutathione [77], which is released from apoptotic cells, promoting an exponential increase of the free payload concentration in the tumor environment. However, despite the high dose (43 mg/kg) and the frequent administration schedule, no complete responses were observed, suggesting that more potent cytotoxic payloads should be used. Indeed, the maytansinoid DM1 payload led to the generation of more potent ADC products based on the F8 antibody [78]. As for cemadotin, DM1 was connected to the SIP(F8) antibody through a cleavable disulfide linker and administered to immunocompetent mice bearing different cancer models (e.g., F9 teratocarcinomas and CT26 colon carcinoma). When administered in three doses of 7 mg/kg, the SIP(F8)-SS-DM1 ADC cured 60% of the treated mice bearing F9 tumors, but not mice bearing the CT26 carcinoma model. These data reflected the 100-fold higher *in vitro* cytotoxicity of free DM1 against F9 cells, as compared to the CT26 cell line. This correlation between the *in vitro* and *in vivo* observations suggested that the tumor cells, rather than the endothelial cells, may be the primary target for the activity of the ADC, despite the selective expression of the EDA antigen around tumor blood vessels.

Coupling of the DM1 payload to F8 did not alter biodistribution profiles when the antibody was used in IgG or SIP format. However, the stability of disulfide linkers was substantially longer for ADC products based on the IgG format [79]. The longer residence time of IgG(F8)-SS-DM1 in the tumor did not result in better anti-cancer properties. A comparative evaluation of IgG(F8)-SS-DM1 and SIP(F8)-SS-DM1, administered to tumor-bearing mice in equimolar doses, revealed a more potent anti-cancer activity for the ADC product in SIP format, even though the IgG product exhibited a slower clearance and a higher tumor accumulation. These data suggest that a suitable (i.e., not too slow) rate of drug release in the tumor environment may be beneficial, in order to expose malignant cells to sufficiently high concentrations of the cytotoxic agent.

The F16 antibody, specific to the A1 extra-domain of tenascin C, has been also investigated as vehicle for cytotoxic agents in both IgG and SIP format. In particular, the antibodies were equipped with the microtubule-disrupting agent monomethyl auristatin E (MMAE) and the protease-sensitive linker Val-Cit [80]. Also in this case, the IgG antibody showed higher absolute accumulation in three different tumor models (A431, U87 and MDA MB 231) as compared to its SIP counterpart. The latter product, however, displayed better tumor/organ ratios, as a result of an efficient tumor uptake combined with a rapid clearance from blood and normal tissues. The administration of IgG(F16)-Val-Cit-MMAE led to complete tumor eradication in mice, bearing either A431 or U87 human tumors. Mice treated with SIP(F16)-Val-Cit-MMAE experienced a significant and prolonged tumor regres-

sion, but tumors eventually started growing again. The different anticancer properties of the ADC products based on the two formats may be explained by considering that: (i) the highly stable peptide linker is compatible with the long half-life in circulation of the IgG-formatted ADC; (ii) the IgG shows a higher absolute accumulation in the tumor than the SIP analogue (i.e., %ID/g of ca. 30 and 10 at 24 h, respectively) indicating that large quantities of payload are necessary to achieve complete response. More recently, other peptide linkers have been investigated for the delivery of MMAE from the IgG(F16) antibody, with the Val-Ala sequence showing similar anticancer activity and *in vivo* metabolic profile to the Val-Cit counterpart [81]. The Val-Cit-MMAE module represents the linker-payload combination used in the approved pharmaceutical product brentuximab vedotin (Adcetris™) and in many others ADCs which are currently in clinical development [16]. Historically, the Val-Cit peptide had been designed as a protease-sensitive linker for products based on internalizing antibodies [82]. The linker should be sufficiently stable in circulation, while being efficiently cleaved by certain intracellular proteases (in particular, cathepsin B) after receptor-mediated endocytosis. This mechanism is supported by *in vitro* cytotoxicity data, whereby only antigen-positive cell lines were efficiently killed by the cognate ADC product [83]. However, the evaluation of the F16-Val-Cit-MMAE product revealed that a more complex series of events may occur *in vivo*, involving an extracellular cleavage and release of the linker-payload combinations.

Improving the Potency and Selectivity of Non-internalizing ADC Products

All three moieties in ADC products (antibody, linker and payload) contribute to activity and selectivity. When non-internalizing antibodies are used, lipophilic payloads capable of rapid diffusion through the cell membrane may be preferred. While proteolytic degradation of the antibody moiety may be a release mechanism for internalizing products with non-cleavable linkers, this option does not apply for agents with long residence in the extracellular space [84]. Non-cleavable linkers have gained increasing research interest in the recent past, also in light of the approval of T-DM1 (Kadcyla™), a product that relies on this technology. The use of non-cleavable linkers is, in principle, attractive for very hydrophilic payloads, as one would expect to confine the cytotoxic agent either to the extracellular space (in which it would not be toxic) or to those cells capable of target-based antibody internalization. Unfortunately, *in vitro* experiments provide insufficient information regarding antigen accessibility and accumulation at the tumor site *in vivo*. Quantitative biodistribution experiments may be combined with other investigations (e.g., plasma stability and immunohistochemistry), in order to gain a detailed information regarding the mechanism of action of ADC products. Other structural innovations in the ADC field, such as the use of polymeric linkers, may result compatible with the use of non-internalizing mAbs. These highly functionalized linkers

allow the macromolecule labelling with a large number of cytotoxic payloads (DAR > 10). While high DARs have often been associated to poor pharmacokinetic properties [85], the hydrophilic nature of these biodegradable polymers has shown favorable plasma PK profiles [86]. However, the potential immunogenicity of these highly functionalized structures may represent an important aspect during clinical investigations.

The use of ADC products may benefit from combination with immunostimulatory drugs. In the recent past, immune-mediated cancer treatment has become an important area of pharmaceutical oncology, thanks to the clinical advance of immunological checkpoint inhibitors, immunocytokines, bispecific antibodies, CAR T cells, vaccines and other products. There are different pathways that may lead to cancer cell death, upon exposure to different types of cytotoxic agents. Some drugs are particularly active for dendritic cell activation and in promoting immunogenic cell death. It is still not clear how tubulin drugs promote direct activation of dendritic cells. Anthracyclines and other DNA-targeting cytotoxics have been found to promote the expression of the so-called damage-associated molecular patterns (DAMPs) [87, 88]. When certain markers (e.g., calreticulin, HMGB1, ATP and type I interferon) are released into the extracellular environment by dying cells, they may stimulate dendritic cell maturation and activation, leading to an increased infiltration of CD8$^+$ T cells into the tumor mass, followed by cytotoxic activity. In many instances, immunological check-point inhibitors (e.g., anti-PD-1 antibodies) are used in patients that had progressed after treatment with conventional anti-cancer drugs, but use of pembrolizumab or nivolumab in first line is becoming more and more frequent [89, 90]. The use of ADC products in combination with certain immunotherapeutic agents can lead to synergistic activity, as damage to cancer cells may result in improved antigen presentation (with subsequent recognition by CD8$^+$ T cells) or surface expression of proteins such as MIC-A, which trigger NK cell activation through NKG2D receptors [91, 92]. Specifically, it would be interesting to understand whether non-internalizing ADC products could give significant advantages over internalizing analogues in enhancing the activity of the immunotherapeutic partner. Indeed, considering the more widespread cytotoxic action that non-internalizing ADCs could promote in the tumor microenvironment, a more heterogeneous area of the solid mass could efficiently lead to inflammation and to an increase of the population of infiltrating lymphocytes in the tumor mass. It is now becoming increasingly evident that this process, often described as the conversion of "immunologically cold" tumors into "hot", is a key parameter to extend the efficacy of immunotherapy to a larger number of patients and indications [93].

Conclusions and Outlook

The possibility to develop non-internalizing ADCs is, by now, firmly established, at least at the preclinical level. Splice isoforms of tenascin-C and of fibronectin represent ideal targets for pharmacodelivery applications, but it is possible that other

tumor-associated antigens may be considered [94]. ECM components offer unique opportunities for pharmacodelivery applications, as these targets are often abundant and stable, thus allowing a long residence time of ADC products at the tumor site. The field of ADC research, both for internalizing and non-internalizing products, will continue to face an important scientific challenge, namely the translation of preclinical data into a prediction of efficacy in patients.

In this context, the therapeutic widows of marketed ADC products were found to be much smaller in human patients than in rodents. For instance, while early clinical studies of MMAE-based ADCs reported MTD values between 1.2 and 2.4 mg/kg [95], administrations of Tenfold higher doses are commonly well tolerated in mice. This important aspect is due to several factors (e.g., the different tumor size in mice and humans, the number of antigen copies in the tumor and their accessibility by ADCs, etc.) and it limits the progression of promising ADC candidates through the clinical stages. The pharmaceutical relevance of this "bottleneck" is reflected in the fact that only two ADC products are currently available on the market, whereas more than 40 ADC candidates are currently being investigated in clinical trials [96]. The use of tumor-associated ECM proteins as targets for ADC development takes the internalization process out of the mechanism of action, thus potentially promoting an easier and more rational design of future ADC products.

One of the most challenging issues for future developments in the ADC field relates to the quantification of product uptake in mouse and man, as well as to the comparative evaluation of drug release kinetics in different species. In particular, a quantitative evaluation of the targeting properties of ADCs in human patients is often missing, which negatively impacts on the clinical development of drug candidates. In principle, initial information about antibody biodistribution, pharmacokinetics, tumor targeting properties and interpatient variability could be obtained from microdosing (phase 0) PET clinical studies [73]. However, these trials are normally performed with drug dosages that are substantially lower than the ones used for therapy purposes. A more systematic and accurate use of imaging techniques for the analysis of antibody performances in patients, as well as the real-time monitoring of ADC fragments at preclinical level (e.g. through the labeling of drug and antibody with different radioisotopes) [97], may provide important insights for the optimal pharmaceutical development of targeted cytotoxics.

In summary, while most academic and industrial efforts have so far been devoted to the development of internalizing ADC products, there is a strong rationale for the design and optimization of antibody-drug conjugates, which do not directly internalize into the target cells. ADC products directed against splice isoforms of fibronectin and tenascin-C are particularly attractive, as those targets are abundantly expressed in the majority of solid tumors and lymphomas, while being virtually undetectable in the majority of normal adult tissues.

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