Abstract. Proteases are often overexpressed in tumor cells and/or the stromal compartment and can thus be exploited in tumor therapy to activate cytotoxic prodrugs as, for example, in cytolytic fusion proteins, and for tumor imaging. Specifically, we discuss cathepsin B-activated prodrug conjugates, antibody-directed prodrug therapy, protease-activated peptide–thapsigargin conjugates, protease-activated cytotoxic receptor ligands and other cytotoxic proteins, protease-mediated activation of anthrax toxin, granzyme B as a therapeutic principle in cytolytic fusion proteins, and tumor-imaging based on deregulated proteases.

The human degradome consists of roughly 600 proteases, which are grouped into metallo-, serine, cysteine, threonine and aspartic proteases (1). They regulate physiological processes such as proliferation, migration, cell survival, apoptosis and protein turnover. In tumors, expression of proteases is frequently dysregulated, thus, coopting these physiological processes, which are often based on complex interactions between tumors and their microenvironment (2). Matrix metalloproteinases (MMPs) are a family of proteins consisting of 26 members of secreted and transmembrane proteins (3) which degrade the extracellular matrix and regulate the activity of other proteases, growth factors and receptors, thus modulating signaling pathways often associated with pro- or antitumoral outcome (4). The family consists of collagenases, stromelysins, gelatinases and membrane-type MMPs (5). In the context of tumor biology, MMP2 (gelatinase A), MMP9 (gelatinase B), membrane-type1 protease MMP14, matriptase (membrane-type serine protease 1), as well as cathepsin B, a cysteine protease (6), were thoroughly investigated (5). In addition, the serine protease urokinase (uPA) and its specific receptor (uPAR) control matrix degradation and many other biological processes by conversion of plasminogen into plasmin and by receptor signaling based on the interaction of uPAR with many extracellular protein ligands (7). The deregulation of this system in several types of cancer has a significant impact on prognosis and can serve as a possible target for therapeutic intervention (8-10). Other proteolytic systems deregulated in cancer include kallikrein-related peptidases (11), a disintegrin and the metalloproteinase family of proteinases (ADAMs) (12), as well as several types of cathepsins (6, 13). In the following, we summarize approaches for tumor-specific activation of antitumoral agents capitalizing on deregulated and overexpressed proteases in several types of human cancer. We focus on MMP2, MMP9, MMP14, matriptase, uPA and cathepsin B.

Cathepsin B-activated Drugs

As outlined before, increased expression or altered localization of proteases is typical in many types of cancer (2). Activation of prodrugs through cleavage by cathepsin B is a common principle in pre-clinical and clinical development of anticancer drugs. Cathepsin B is a lysosomal cysteine protease which is overexpressed in numerous tumor types and can be associated with the external cell surface (14). Procathepsin B can be activated by several proteases. Activated cathepsin B can then convert pro-uPA to uPA, which finally activates plasminogen to plasmin, a serine protease, which can degrade several components of the extracellular matrix such as fibrin, fibronectin, laminin and proteoglycans and which is able to activate MMPs (15, 16).

Several strategies for the construction of prodrug therapeutics have been evaluated. A common motif here is the combination of a therapeutic agent with an inactivating peptide substrate which is cleavable by proteases, thus leading to the liberation of the active therapeutic. In addition, more recently,
such prodrug molecules were fused to a targeting moiety. Alternatively, peptide-linked therapeutic agents can also be fused to a macromolecular carrier with or without a targeting moiety (17). The targeting module can, for example, be a ligand for a receptor, or an antibody-based moiety. In the case of cathepsin B-activated prodrugs, conversion into the active drugs should occur in the lysosome, but due to possible association of cathepsin B with the cell surface in some types of cancer, extracellular activation of the substrate is also possible. In addition, non-internalizable prodrug-receptor complexes will be activated exclusively at the cell surface. However, depending on the design of the substrate cleavage site, prodrug activation in the serum by cathepsin B-like activity is a critical issue. In the present review article, we summarize selected approaches for the design of cathepsin B-activated prodrugs, including cytotoxic antibody–drug conjugates (ADCs) which are at an advanced clinical stage.

Linking cytotoxic agents to high-molecular weight carrier molecules is a strategy to deliver drugs to tumors. The resulting modification of the pharmacokinetic properties of the cytotoxic compound mediates tumor accumulation in pre-clinical models, so-called passive targeting (18). For example, FCE 28068 (also known as PK1) was constructed by linking doxorubicin to N-(2-hydroxypropyl) methacrylamide copolymers via a tetrapeptide spacer which is cleavable by cathepsins, allowing release of the active topoisomerase II inhibitor (19). This form of passive tumor targeting is also known as the enhanced permeability and retention effect, resulting from enhanced permeability of the tumor vasculature, facilitating extravasation of high-molecular weight proteins such as drug conjugates from the blood vessels (20). In a phase II study of FCE 28068 in chemotherapy-refractory patients with breast cancer, non-small cell lung carcinoma, and colorectal cancer, 6/62 partial responses were observed with limited side-effects thus supporting the concept that polymer-based chemotherapeutics might have different and improved anticancer activities (21). In addition, FCE 28068 was linked to galactosamine, resulting in drug PK2, which binds to the hepatic asialoglycoprotein receptor, thus achieving liverspecific doxorubicin delivery (22). Indeed, in a phase I study, liver-specific accumulation of PK2 after i.v. infusion was shown, as well as targeting to primary hepatocellular tumors (22). Similarly, the tubulin-binding compound paclitaxel–polyglumunex (PPX) is a polymer–drug conjugate in which paclitaxel is linked to a degradable polymer consisting of L-glutamic acid residues. The release of paclitaxel from the polymer backbone is dependent on cathepsin B and other lysosomal proteases (23). PPX was evaluated in a phase III trial versus docetaxel in second-line treatment of patients with non-small cell lung cancer (24, 25). Although PPX and docetaxel led to similar survival rates, different toxicity profiles were noted. Compared to docetaxel, PPX-treated patients had less frequent febrile neutropenia and alopecia, however, increased neurotoxicity was noted for PPX in comparison to docetaxel.

ADCs consist of a targeting moiety, a linker, and a cytotoxic payload. The targeting module can be a ligand for a receptor or an antibody-based moiety directed against a cancer-related antigen (26). After internalization of the ADC antigen–receptor complexes, cytotoxic payloads such as calicheamycin, maytansine, duocarmycin, auristatin or irinotecan are released into the cytosol after cleavage of the linker in the endosome (27). Chemically-unstable, enzyme-cleavable, but also stable, non-cleavable linkers have been explored in this context (26). Chemically labile linkers are hydrazine-based, whereas stable, non-cleavable linkers make use of thioester chemistry (26). The activity of ADCs with stable, non-cleavable linkers seems to depend on the degradation of the thioester-linked antibody component in the lysosome, resulting in the release of the cytotoxic payload (28). Molecules with enzyme-cleavable linkers, on the other hand, rely on the hydrolysis of a valine-citrulline (vc) linker by cathepsin B. This results in the release of the cytotoxic payload, such as the tubulin binders monomethyl auristatin E (MMAE) and F (MMAF) (29, 30, 31). ADCs based on cathepsin B cleavable linkers are among the most advanced ADCs for the treatment of cancer. For example, brentuximab vedotin, an IgG1 antibody directed against cluster of differentiation-30 (CD30) which is linked to vc MMAE is approved for treatment of therapy-refractory Hodgkin’s lymphoma and anaplastic large cell lymphoma (32, 33). Similarly, CDX-011 (glembatumumab vedotin) is an ADC composed of a fully human IgG2 anti-glycoprotein non-metastatic melanoma protein b/osteoadcin (gpNMB) antibody conjugated to MMAE via a vc linker (34). CDX-011 is presently in phase II clinical studies in melanoma and breast cancer (35-36). In addition, a prostate-specific membrane antigen (PSMA)-ADC composed of a fully-humanized immunoglobulin G1 (IgG1) antibody and MMAE linked via a vc linker and is currently being evaluated in patients with taxane-refractory metastatic prostate cancer in phase I clinical trials (37, 38). ADCs with analogous design targeting nectin-4 and solute carrier family 4A4 (SLC4A4) are currently being evaluated in phase I clinical studies (27). Also undergoing phase I clinical studies are RG-7458, targeting CD22 in patients with non-Hodgkin’s lymphoma, and SGN75 and MDX 1203, two ADCs based on anti-CD70 antibodies. These are presently being evaluated in patients with non-Hodgkin’s lymphoma and clear cell renal cancer (27). In summary, it seems that no general design for optimized linkers can be deduced based on previous experience. In addition, ADCs with non-cleavable linkers must be efficiently internalized to exert their cytotoxic function. On the other hand, ADCs with cleavable linkers may mediate cytotoxicity by extracellular cleavage at the tumor cell surface in addition to cytotoxic pathways. Therefore, toxicity directed against antigen-negative tumor cells is expected only for ADCs with cleavable linkers (26).
Antibody-directed Enzyme Prodrug Therapy (ADEPT)

ADEPT is based on pre-targeting antibody–enzyme complexes or corresponding fusion proteins to tumors, followed by administration of an inactive prodrug which is converted to an active drug on the tumor cell surface by these enzymes (39-41). After administration of the antibody-enzyme conjugates, the unbound conjugate is allowed to be cleared from the circulation. This can be accelerated by using mannosylated conjugates (42), or a second antibody (43). For effective treatment, the prodrug has to be an optimal and specific substrate for the enzyme and must not be able to penetrate cells. The inhibitory concentration 50 (IC50) of the liberated drug should be below 10 nM, and the ratio of the IC50 of the prodrug and drug should exceed 1,000 (40). The released drug must be able to penetrate tumor cells and its plasma half-life should be short enough to prevent leakage into healthy tissues, but long enough to induce a bystander effect on neighbouring tumor cells. Activation of prodrug can be mediated by proteases, glycosidases, alkaline phosphatase, penicillin amidase, β-lactamase or cysteine deaminase (41).

In the following, we focus on prodrug activation by proteases. Most thoroughly explored, also in terms of translational applications in clinical studies, is carboxypeptidase G2 (CPG2). CPG2 is a bacterial, Zn2+-dependent, homodimer forming exopeptidase, which can cleave a deactivating glutamate moiety from benzoic acid mustard (42, 43). Initial pre-clinical and clinical studies were performed with chemically produced antibody–enzyme conjugates. Subsequently, in order to overcome the variability of random chemical conjugation which results in product heterogeneity, recombinantly produced antibody moiety–enzyme fusion proteins were explored (42, 43). Such a fusion protein consisting of a single-chain variable domain fragment (scFv) directed against carcinoembryonic antigen (CEA), and CPG2 produced in Pichia pastoris for activation of a methotrexate-based prodrug was investigated in more detail. This fusion protein, MPECP, bore branched mannose residues due to post-translational N-glycosylation (44). However, despite rapid clearance via mannose receptors, tumor-to-plasma ratios of 1,400:1 in the LS174T colon xenograft model and 339:1 in the SW122 colon xenograft model were recorded (45). Since the fusion protein localized specifically to CEA-expressing xenografts in the mouse, the prodrug was administered 6 h after administration of the fusion protein. Cycles could be repeated without obvious toxicity (45). Consequently, this concept was explored in clinical studies in heavily pre-treated patients with CEA-expressing tumors. Treatment resulted in stable disease eight weeks after therapy in 11 out of 28 patients (46). Three cycles of treatment could be applied after administration of cyclosporine to suppress immune responses against the fusion molecule (47). For second-generation approaches, humanized antibodies and CPG2 with removed B- and T-cell epitopes are under consideration (48).

Protease-cleavable Peptide–Thapsigargin (TG) Conjugates

The peptide–TG drug platform is based on coupling a chemically modified amino acid containing TG with protease-cleavable peptides (49). TG is a sesquiterpene lactone isolated from the plant Thapsia garganica (50). TG is a lipophilic molecule and an inhibitor of the ubiquitously expressed sarcoplasmic/endoplasmic reticulum (ER) calcium ATPase pump (SERCA) (51, 52). The SERCA pump is essential for the function of the ER. SERCA inhibition leads to a 3-5 fold increase in intracellular free Ca2+ accompanied by the depletion of the ER Ca2+ pool resulting in cell death independent of proliferation status (53, 54). Because the toxicity of TG is not cell type-specific, systemic administration of TG would be associated with significant toxicity. Therefore, prodrug molecules were designed with a carrier peptide that does not allow TG to enter the cell until the TG analog is liberated by proteolysis. A PSMA-targeted prodrug was evaluated (55). PSMA is a membrane-bound protease with its catalytic domain exposed to the extracellular space. It exhibits dual exopeptidase activity as both a pteroly pol γ glutamyl exopeptidase (folute hydrolase) and N-acetylated α-linked acid dipeptidase (56, 57). PSMA is expressed in tumor-associated endothelium, thus potentially enabling TG prodrg activation in the tumor microenvironment (53). The TG-based prodrug G202 (Figure 1A), consisting of a PSMA-specific peptide conjugated to an analog of TG, induced tumor regression in several xenograft models at doses which were minimally toxic to the host (55). Critical issues are, however, the expression of PSMA in the proximal tubules of the kidney and in the brain (58). Ongoing clinical studies will show whether a therapeutic window combining efficacy with an acceptable toxicity profile can be defined.

A new emerging strategy for tumor therapy is ablation of supporting stromal cells in the tumor microenvironment by targeting cancer-associated fibroblasts (59). Fibroblast activation protein (FAP), a post-prolyl endopeptidase predominantly expressed in cancer-associated fibroblasts with a limited pattern of expression in normal tissues has emerged as an interesting target (60). A FAP-activated peptidyl-TG prodrug killed FAP-positive human cancer cells at low nanomolar concentrations, efficiently reduced growth of MCF-7 and LNCaP tumor xenografts, concurrent with ablation of tumor stroma (61). However, activation of the prodrug in plasma was observed indicating a possible limitation of this approach (61). Ongoing efforts are based on activation of TG-based prodrugs by prostate-specific antigen and human kallikrein-2, proteolytic enzymes secreted by the prostate with expression orders of
molecules. These prodrugs are composed of an scFv-directed sensitive linker which can be cleaved by MMP-2 and related proteases and the ECD of CD95 as carboxyterminal inhibitory domain (Figure 1B and C). In order to achieve tumor-specific activation of CD95, CD95L is only unmasked after binding of the fusion protein to FAP-positive tumor cells and subsequent cleavage of the linker by tumor-associated proteases. The cleaved scFv-CD95L remains membrane-bound and thereby mimics membrane CD95L. It was shown that activation of CD95L in vitro is dependent on CD95 binding and protease-dependent processing. In vivo efficacy was demonstrated by intra-tumoral injection of FAP-transfected HT1080 cells together with the prodrug one day after tumor inoculation with $1.5 \times 10^6$ cells. This resulted in 80% tumor growth inhibition which was not observed when non-transfected HT1080 cells were co-injected. A critical issue of the approaches, as outlined, is the potential activation of the fusion proteins by serum proteases. Therefore, efficient targeting of the tumor with these effector fusion moieties is mandatory because expression of tumor-associated proteases is not strictly tumor-specific.

Mellitin is a 26 amino acid pore-forming protein derived from honey bee venom (72). Formation of pores in the cell membrane induces cell lysis. In order to target the cytotoxic activity of mellitin to MMP2-overexpressing tumor cells, a biologically-inactive fusion protein consisting of mellitin and avidin connected by an MMP2 cleavage site was designed (73). This fusion protein mediated strong cytotoxic activity after activation by MMP2 in MMP2-overexpressing tumor cells, while only little cytotoxicity was seen in cells with low MMP2 activity. In vivo tumor shrinkage was also shown.

Fusogenic membrane glycoproteins were identified in several viruses inducing cell-cell fusion and syncytia formation, which ultimately results in cytotoxicity and cell death (74). The Gibbon Ape Leukemia Virus envelope glycoprotein (GALV) was assessed for its potential for targeted killing of glioma cells. Thus, GALV was linked to the extracelluar domain of CD40 ligand as a binding blocking moiety, via an MMP or factor Xa protease-cleavable linker, or a non-cleavable linker. Transfection of constructs with cleavable linkers selectively induced cytotoxicity in glioma cells in contrast to non-cleavable constructs (75). Targeting in this case is necessary due to the ubiquitous expression of the GALV receptor, pituitary-specific positive transcription factor-1 (PTF-1). Adenoviral vectors expressing fusion proteins, as outlined above, were shown to exhibit significant antitumoral potential for gene therapy of glioma gene therapy (76).

**Protease-mediated Activation of Anthrax Toxin for Tumor Therapy**

*Bacillus anthracis*, a bacterium that forms highly resistant spores, can cause lethal infections after inhalation or ingestion. The causative agent for lethal infections is anthrax toxin which consists of three components which individually are non-toxic: protective antigen, lethal factor (LF) and edema factor (EF),...
The unique processing and assembly of the components of the anthrax toxin was exploited in the design of anti-tumoral agents and for the delivery of other toxins into tumor cells. The action of anthrax toxin starts by binding of protective antigen to two broadly-expressed receptors: tumor endothelial marker-8 and capillary morphogenesis protein-2 (77). After binding to its receptors, protective antigen is processed by proteases such as furin, and the remaining receptor-bound moiety of protective antigen forms a ring-shaped heptamer which transports EF, LF, or both of them into the cell to exert their cytotoxic function. The resulting complexes are referred to as lethal toxin or edema toxin. These complexes are internalized by receptor-mediated endocytosis via a clathrin-dependent process, and after formation of a

Figure 1. Display of G202 (A) and cluster of differentiation 95 ligand (CD95L)-based (B) prodrugs. A: Schematic presentation of G202. The toxic entity thapsigargin (orange) is fused via an aliphatic linker (pink) to a cleavable prostate specific membrane antigen (PSMA)-specific peptide (grey). Protease cleavage sites are indicated by arrows. B: CD95L-based prodrug. The proteins are displayed as ribbons coloured according to domain function. The CD95L prodrug is composed of the CD95 receptor (magenta, pdbcode 3TJE) as the protecting moiety, followed by a trimerization coiled-coil domain of tenasin C (shown in green, helix modeling is based on pdbcode 2F3M), a cleavable linker (grey), a modeled scFv directed against fibroblast activation protein (FAP) (sc FAP) heavy and light variable domains displayed in dark and light blue and their corresponding linker shown in pink, a polyaspartate flag (red) and finally the CD95L domain as an effector (orange, pdbcode 4M5V). The lower left shows the cyclic configuration of the CD95L-based prodrug, bringing the ligand and the corresponding receptor together. The protease cleavage site is indicated by an arrow. The trimeric prodrug is shown in the middle and lower right. The head-to-tail configuration is not shown. The models were generated based on available structural data of domains or entities and were assembled and minimized using DiscoveryStudio40 (128, 129).
channel in the membrane of the endosome, LF and EF are transported into the cytosol. LF is a zinc-dependent metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKK). EF is a calmodulin-dependent adenylate cyclase which leads to intracellular elevation of adenosine-monophosphate or cyclic adenosine monophosphate levels, thus interfering with the balance of intracellular signaling (79, 80). Anti-angiogenic effects were observed for both LF and EF due to their effects on MAPKK inhibition and cAMP levels in endothelial cells. LF interferes with MAPK signaling in Ha-ras transformed NIH-3T3 cells, inhibits their growth in soft-agar, reverts their transformed phenotype and inhibits their growth in athymic mice, partly through inhibition of angiogenesis (81). Human melanoma cells were shown to be equisitely sensitive to LF in combination with a small molecule MAPK inhibitor, inducing apoptosis, whereas normal melanocytes were not killed but arrested in the G1-phase of the cell cycle (82). Due to the broad expression of the receptors, tumor-specific targeting of anthrax toxin is essential to generate a therapeutic window. Activation of modified anthrax toxins on the surface of tumor cells is a straightforward strategy. MMPs such as MMP2, MMP9, MMP14 and matriptase, as well as uPA are proteases which are overexpressed on the surface of tumor cells or the surrounding stromal cells. Replacement of the furin cleavage site in protective antigen by those specific for MMPs or uPA leads to tumor-specific activation of protective antigen. Making use of MMP-activated LF, antitumor activity was observed in tumor xenograft models harbouring the V600E mutation, but also in xenografts with non-mutated BRAF (83). In addition, anthrax toxin inhibits tumor angiogenesis by interference with endothelial cell proliferation, migration and tube formation for which MAPK activation plays a crucial role. Delivery of other toxin effector moieties such as the ADP-ribosylation domain of pseudomonas exotoxin into tumor cells making use of the properties of the anthrax toxin complex is another potential application of this system. Thus, fusion of passenger proteins with amino acids 1-254 of LF allows their translocation into, and eventually killing of cells expressing PA receptors with toxic effector molecules such as the ADP-ribosylating moiety of pseudomonas exotoxin, the A subunit of diphtheria toxin, and the A subunit of Shiga toxin (84, 85, 86). Similarly, when amino acids 1-254 of LF were combined with mutated protective antigen proteins, the resulting fusion protein (FP59) mediated selective killing of MMP-overexpressing tumor cells such as fibrosarcoma, breast and melanoma (83). Analogous observations were made with uPA activated protective antigen which resulted in selective killing of uPAR-expressing tumor cells (87). A critical issue is that neither the MMP nor uPA-activation systems are strictly tumor-specific, thus side-effects after administration of these agents are an issue.

**Granzyme B as Effector Moiety for Cytolytic Fusion Proteins**

Receptor–ligand or antibody-based moieties directed against tumor antigens genetically fused to granzyme B as a cytotoxic principle are presently pre-clinically evaluated as anti-tumor agents (88-92). Granzyme B is member of a family of serine proteases with five different human granzymes (A, B, H, K and M). They are stored in the granules of natural killer cells and cytotoxic T-lymphocytes. Granzyme B is an inducer of granule-mediated apoptosis. Granzyme B cytolytic fusion proteins as described above are internalized and induce apoptosis after release from the endosome. Once in the cytosol, granzyme B can directly activate different apoptotic pathways and at different levels, ensuring induction of apoptosis even when some apoptotic pathways are blocked. Important granzyme B substrates include caspase-3 and other initiator and effector caspases, the inhibitor of caspase-activated DNase, and BH3-interacting domain death agonist (BID). In addition, granzyme B can cleave further death-substrates such as poly(ADP ribose)polymerase, DNA-dependent protein kinase, components of the cytoskeleton and the nuclear mitotic apparatus as well as proteins involved in stress response and cellular homeostasis. Numerous variations of granzyme B fusion proteins were studied. Transforming growth factor-α and vascular endothelial growth factor (VEGF) (Figure 2A) were the targeting domains in the context of ligand–granzyme B fusions, while CD64, GP240, Lewis Y antigen, human epidermal growth factor receptor 2 (HER2) and human luteinizing hormone receptor were evaluated as targets for antibody-based moieties of granzyme B-derived cytolytic fusion proteins (88). Despite encouraging *in vitro* and *in vivo* efficacy data in various tumor-derived cell lines and xenograft models, several problems preventing optimal efficacy of such granzyme B-based cytolytic fusion proteins have emerged: (i) granzyme B inhibitor PI-9 is expressed in several tumor types and their corresponding cell lines, limiting efficacy; and (ii) granzyme B possesses a strong positive surface charge which causes non-specific binding to negatively charged cell surfaces. This leads to adsorption and off-target effects, as well as trapping of the internalized antigen–fusion proteins in endosomes, resulting in insufficient delivery of granzyme B into the cytosol. Consequently, highly variable efficacy of the corresponding cytolytic fusion proteins was observed. One recent achievement is the generation of granzyme B mutants insensitive to inhibition by PI-9 by mutating positions interacting with the variable reactive center loop of PI-9, which is its primary site of interaction with proteases. Importantly, one of these variants, granzyme B R201K, exhibits the same enzymatic activity in the absence or presence of PI-9 (93). Mutation of sequences responsible for interaction with heparan-sulfate-containing molecules resulted in diminished cell binding but had a negative impact on cytotoxicity (94).
Residues 96-103 and 221-226 confer a positive surface charge on mature granzyme B, causing interactions with glycosaminoglycans. Substitution of such cationic residues was shown to have no impact on the enzymatic and pro-apoptotic activity of granzyme B but reduced binding to antigen-negative cells (95). Improved efficacy of granzyme B-based cytolytic fusion proteins was achieved by co-administration with chloroquine, which accumulates in acidic compartments and lysosomes and causes osmotic rupture of these vesicles (95-97). Further approaches for improved endosomal release are the incorporation of (i) protein transduction domains derived from pseudomonas exotoxin or diphtheria toxin; (ii) cell-penetrating peptides or membrane transfer sequences into the corresponding cytolytic fusion proteins; and (iii) insertion of synthetic, multifunctional linkers, as outlined below (98). These linkers are composed of a membrane transfer peptide flanked by an endosomal cleavable peptide and a cytosolic cleavable peptide (98). Potential immunogenicity after introduction of amino acid substitutions has not yet been properly investigated. An interesting example for the improvement of efficacy is a fusion protein between a scFv directed against HER2, a fusogenic pH-sensitive peptide, and granzyme B (99). Tumor cells resistant to lapatinib or Herceptin and expressing MDR1 showed no crossresistance to the granzyme B-based fusion protein.

Figure 2. Display of a targeted granzyme B-based fusion protein and two different versions of a probody. A: Granzyme B-vascular endothelial growth factor 121 (VEGF121) fusion protein. The model is based on the structures of granzyme B (orange, pdbcode 3PW9) and VEGF121 (blue, pdbcode 1FQ4). The models were generated based on available structural data of domains or entities and were assembled and minimized using DiscoveryStudio40 (128, 129). B: Schematic presentation of a probody. The principle design is derived from a probody directed against vascular cellular adhesion molecule 1 (VCAM1) (117). The peptide with lid function (magenta) has affinity to the antibody paratope. Dark and light orange variable domains were taken from a non-related Fab complex (pdbcode 4RNRX), the peptide is linked via a flexible glycine-serine linker (pink) and a cleavable sequence (grey) to the heavy chain variable domain (VH domain). The constant part of the antibody is shown in grey. Protease cleavage sites are shown in grey. C: Schematic presentation of a bi-specific probody. Targeting to antigen 1 is mediated by the V domains shown in dark and light blue. The molecule is based on the knob-into-hole differentiation of the two fragment crystallization region (Fcγ), highlighted in dark and light grey. V-domains of an antibody directed against antigen 2 are fused to the C-termini of the Fc portions by connectors with and without a protease cleavage site. VH and VL domains are disulfide bridged. Binding functionality is reconstituted after cleavage of one of the two linkers. The cleavable element is shown in grey and G4S in pink. The schematic presentation is based on the description of a bi-specific prodbody directed against human epidermal growth factor receptor 3 (HER3) and cellular proto-oncogene of mesenchymal epithelial transition factor (c MET) (120). The protease cleavage site is indicated by an arrow and the disulfide bridge connecting VL and VH domains is shown in green.
Protease-cleavable Adapters for Improved Cytolytic Fusion Proteins

Cytolytic fusion proteins are composed of a targeting module and a protein-based cytotoxic moiety. The targeting module can be a ligand for a cell surface receptor or an antibody-based moiety, the therapeutic principle as a rule is an enzyme with cytotoxic properties. The concept of cleavable adapters was raised (98, 100) to improve the characteristics of cytolytic fusion proteins. The prototype adapter is composed of a cytosolic cleavable peptide, a cell-penetrating peptide (CPP) which is a membrane transfer peptide, and an endosomal cleavable peptide (98). For example, a cytosolic cleavable peptide was designed by incorporating cleavage sites for caspases 1, 3 and 7 and a cytosolic protease from yeast into a corresponding peptide sequence (101). After cleavage, the compound is trapped within the cell, converting a formerly cell-permeable compound into a cell-impermeable drug. CPPs can penetrate membranes by an energy-independent process or by an endocytosis-related process (102, 103). CPPs are of diverse origin, 10-16 amino acids long and can be grouped into peptides composed of basic amino acids such as polylysine or poly-arginine, α-helical, and β-sheet peptides (102, 103). Examples are TAT from HIV, penetratin from Drosophila homeotic transcription factor antennapedia, or the preS2-domain of hepatitis B virus surface protein (104). The function of membrane transfer peptides and CPPs is to improve cellular uptake and deliver the cytotoxic molecule directly into the cytosol. Endosomal cleavable peptides on the other hand are based on dedicated translocation sequences which are activated after protease cleavage and usually are derived from diphtheria toxin or pseudomonas exotoxin (105).

One exemplary adapter was designed to improve the uptake and transport of the catalytic cytotoxic moiety into the cytosol and subsequently trap it in the cell. This trapping might be responsible for the reduced cytotoxicity which was associated with long circulation times and damage of target-negative cells (98). Saporin linked to EGF with or without adapter was evaluated, and reduced non-specific cytotoxicity was noted by inserting a cleavable adapter, as outlined (101). Similarly, treatment of human EGFR-transfected murine tumor cells with adapter-containing and adapter-free fusion protein showed enhanced efficacy for the adapter-containing fusion protein (106). Surprisingly, however, in vitro cytotoxicity was not affected by the insertion of an adapter (101). Another example is a fusion protein between a humanized scFv directed against CD64 and human ribonuclease angiogenin (107). Insertion of an adapter improved cytotoxicity 20-fold, however, serum stability was reduced, resulting in complete cleavage of the adapter after 1 h, whereas the adapter-free fusion protein was stable with no evidence for cleavage after 24 h. Consequently, a fusion protein bearing a modified adapter with deleted endosomal cleavable peptide was evaluated. Thus, while cytotoxicity of this molecule was still 10-fold better than the adapter-free fusion protein, almost no cleavage was observed after 24 h in the presence of serum.

Finally, the findings as outlined above indicate that an adapter cannot be transferred between fusion proteins by default to improve cytotoxicity and stability but has to be optimized specifically for each fusion protein under consideration.

Activation of Antibodies with Impaired Functionality by Proteases

Antibody-based therapies often exploit targets which play a crucial role in physiological processes and therefore can be associated with serious toxicities (108). For example, blockage of delta-like ligand 4, a notch ligand, leads to inhibition of angiogenesis in pre-clinical models. However, liver pathology, gut and T-cell toxicity, and induction of vascular neoplasms were observed in mice and rats (109, 110, 111). Similarly, the anti-angiogenic agent bevacizumab, a VEGF-neutralizing monoclonal antibody, can cause side-effects such as hypertension, proteinuria and gastrointestinal symptoms, and in rare cases, gastrointestinal perforation and arterial thromboembolic complications (112). Skin toxicity as dose-limiting toxicity was observed after treatment of cancer patients with antibodies or antibody conjugates directed against EGFR (113, 114). An increased risk of serious infection and a dose-dependent risk for malignancies was observed in patients with rheumatoid arthritis after anti-TNF antibody therapy (115). HER2-directed therapy with trastuzumab caused an incidence of 0.2-3.8% of class III congestive heart failure in the adjuvant trastuzumab trials of patients with HER2-positive breast cancer. This was probably due to the expression of HER2 on cardiomyocytes in addition to tumor tissues (116). This list of serious target-related side-effects could be extended to numerous other examples. Therefore, the probody concept, activation of an inactive preform of an antibody by tumor-associated proteases was raised (117) (Figure 2B). Accordingly, a probody with masked antibody binding site was designed by tethering a peptide to the N-terminus of an antibody-derived binding domain via a flexible linker containing a protease-cleavage site in the context of a scFv-Fc format (117). In a proof-of-concept experiment, such a probody directed against vascular cell adhesion molecule-1, a marker of atherosclerotic plaques, was evaluated (117). In vitro activation of the probody with MMP1 resulted in a 200-fold increase of binding affinity and restored binding to tissue sections from atherosclerotic mice ex vivo demonstrating MMP-dependent probody
accumulation in aortic plaques. Analysis of frozen aorta tissue sections from mice injected with such probodies revealed large plaques that stained strongly for the presence of the antibody to vascular cell adhesion molecule-1 and probody. Regarding possible oncological applications of the probody technology, antibodies against matriptase were successfully used for imaging of colon cancer xenografts, pointing to matriptase as a possible activator of probodies (118). Tumor-specific activation of therapeutic antibodies potentially results in an improved therapeutic index (119). For example, the EGFR-targeting antibody cetuximab was converted into a probody by fusing a masking peptide which blocks binding to EGFR to the N-terminus of the L-chain. The masking peptide (21 amino acids) was fused to a cleavable linker (26 amino acids), sensitive to uPA, matriptase, and legumain. This probody possessed in vitro activity equivalent to cetuximab, was activated ex vivo by xenograft tumors, and suppressed tumor growth in vivo in mouse xenograft models with efficacy equivalent to cetuximab. However, due to the lack of crossreactivity of cetuximab with rodent EGFR, final conclusions are limited. This probody was shown to be activated by human tumor samples in vitro. The probody was nearly inert in the circulation and possessed markedly improved safety with respect to cutaneous toxicity and increased plasma half-life in non-human primates. Therefore, this probody could be dosed safely at much higher levels than cetuximab (119). Finally, proof-of-concept experiments have shown that bi-specific antibodies with restricted binding functionality can be activated by proteolytic processing (120). The principal design of such a bi-specific probody is shown in Figure 2C. A bispecific antibody which binds HER3 in a conventional, bivalent IgG-like manner and which carries in addition a cellular proto-oncogene of mesenchymal epithelial transition factor (C-MET) binding moiety composed of a heavy weight chain (VH) and a variable light chain (VL)-domain which can be connected by a disulfide bond was designed (120). The H- and L- chain domains are linked to the C-termini of the constant heavy chain 3 domains, respectively. One of the connectors contains a protease cleavage site for MMP2, MMP9 or uPA. After cleavage of the protease-sensitive connector, steric hindrance is resolved and antigen access and affinity in vitro is fully restored.

Proteases and Imaging

Tumor-associated expression of enzymatically active matriptase has been exploited for tumor imaging (122). Matriptase is a type II transmembrane protease of the serine protease family. It is found on the surface of epithelial cells and its activity is tightly regulated by its cognate inhibitor, hepatocyte growth factor activator inhibitor-1. In normal tissues, the ratio of matriptase to its inhibitor is low. This ratio increases during progression of some types of cancer, resulting in active matriptase on the cell surface (121, 122). Using a recombinant human antibody against an epitope covering the active site of matriptase, it was shown that tumor epithelium can be visualized selectively (123, 124). Live cell fluorescence imaging showed that the antibody localized only to the surface of matriptase-positive tumor cells, and immunofluorescence experiments with tissue microarray sections from primary and metastatic colon cancer indicated active matriptase in 68% of primary and metastatic colon cancer specimens (122). Near infrared and single-photon emission computed tomographic imaging visualized matriptase in human colon cancer tumors in a patient-derived xenograft model (122). The antibody used in these studies was crossreactive with the murine matriptase ortholog epithin, and radiolabeled antibody was taken-up by tumor xenografts. Specificity of the interaction was demonstrated by inhibition of uptake of the antibody after injection of ecotin, a macromolecular inhibitor of matriptase, before injection of the radiolabeled antibody. Accordingly, active site probes may become important tools for the elucidation of the role of proteases in the pathogenesis of different types of cancer.

Concluding Remarks

Personalized medicine is one of the key issues of cancer therapy (125-127) meaning that patients are treated based on molecular characteristics as revealed by diagnostic tests and/or high throughput sequencing. Overexpressed plasma membrane-associated receptors, oncogenic driver mutations and other deregulated cancer promoting pathways are targets for small molecule- or antibody-derived therapeutics, resulting in inhibition of migration, induction of apoptosis, inhibition of angiogenesis, stimulation of an antitumoral immune response, or synthetic lethality. Knowledge about, and specification of, the expression pattern of proteases in tumors, and quantification of the ratio of inactive proenzymes versus enzymatically active ones in individual tumors could advise treatment with prodrugs activated by the proteases characteristic of the individual tumors. Finally, these findings will also have an impact on optimal tumor imaging. A further future application is the administration of cytolitic fusion proteins with proteases such as granzyme B as an effector moiety.

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