REVIEW ARTICLE

Applying Antibodies Inside Cells: Principles and Recent Advances in Neurobiology, Virology and Oncology

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Abstract

To interfere with cell function, many scientists rely on methods that target DNA or RNA due to the ease with which they can be applied. Proteins are usually the final executors of function but are targeted only indirectly by these methods. Recent advances in targeted degradation of proteins based on proteolysis-targeting chimaeras (PROTACs), ubiquibodies, deGradFP (degrade Green Fluorescent Protein) and other approaches have demonstrated the potential of interfering directly at the protein level for research and therapy. Proteins can be targeted directly and very specifically by antibodies, but using antibodies inside cells has so far been considered to be challenging. However, it is possible to deliver antibodies or other proteins into the cytosol using standard laboratory equipment. Physical methods such as electroporation have been demonstrated to be efficient and validated thoroughly over time. The expression of intracellular antibodies (intrabodies) inside cells is another way to interfere with intracellular targets at the protein level. Methodological strategies to target the inside of cells with antibodies, including delivered antibodies and expressed antibodies, as well as applications in the research areas of neurobiology, viral infections and oncology, are reviewed here. Antibodies have already been used to interfere with a wide range of intracellular targets. Disease-related targets included proteins associated with neurodegenerative diseases such as Parkinson's disease (α-synuclein), Alzheimer's disease (amyloid-β) or Huntington's disease (mutant huntingtin [mHtt]). The applications of intrabodies in the context of viral infections include targeting proteins associated with HIV (e.g. HIV1-TAT, Rev, Vif, gp41, gp120, gp160) and different oncoviruses such as human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV) and Epstein-Barr virus, and they have been used to interfere with various targets related to different processes in cancer, including oncogenic pathways, proliferation, cell cycle, apoptosis, metastasis, angiogenesis or neo-antigens (e.g. p53, human epidermal growth factor receptor-2 [HER2], signal transducer and activator of transcription 3 [STAT3], RAS-related RHO-GTPase B (RHOB), cortactin, vascular endothelial growth factor receptor 2 [VEGFR2], Ras, Bcr-Abl). Interfering at the protein level allows questions to be addressed that may remain unanswered using alternative methods. This review addresses why direct targeting of proteins allows unique insights, what is currently feasible in vitro, and how this relates to potential therapeutic applications.

Key Points

Therapeutic antibodies are valuable drugs, which mostly act outside of cells.

Reaching the numerous drug targets that reside inside cells by antibodies is possible in vitro and allows unique insights compared with other methods.

Applying antibodies inside cells for therapeutic purposes has been explored in animal models and promises specific therapeutic benefits in neurobiology, virology and oncology.

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1 Why Target Proteins Directly?

Targeting proteins inside cells can serve the development of therapies in two ways: by using antibodies as drugs themselves inside cells or by using them as a tool to characterize and understand protein components of signalling pathways and thus identify targets for the development of new drugs. The hurdle for applying antibodies as drugs inside cells is often their delivery into the cell, which has so far been considered the "high-hanging fruit", as detailed by Carter and Lazar [1]. Although DNA, RNA and proteins can all be introduced into cells, they may require different delivery methods. Delivering proteins to the cytosol is challenging, and progress has been hampered by inappropriate detection methods that substantially overestimated delivery efficiency. The importance of appropriate assays and scrutiny

of evaluation criteria when interpreting the results of protein delivery experiments has since become evident [2–4].

Targeting at the protein level offers the advantages of targeting protein conformations, post-translational modifications, splice variants, different functional epitopes, targeting individual cellular compartments and interference independently of protein half-life (see details in Table 1). In contrast to knocking out a gene, interference at the protein level furthermore allows tuning the amounts of a target protein instead of allowing only the presence or complete absence of the protein [5, 6]. The rapid effects achievable by protein delivery can be used to interfere in a stage-specific way and when time is a relevant factor. This is also relevant if the time required to obtain a single clone with a gene knockout is long enough to allow genetic compensation for the modification introduced [6, 7]. Delivering proteins directly circumvents stable DNA integration into the genome. The temporally more limited presence of proteins than that of permanently integrated DNA might also allow better control over therapeutic effects. Direct protein delivery is also a suitable method for inducing effects in non-dividing cells [8]. Furthermore, protein mutations could be specifically targeted and distinguished from the wild-type version, such as mutated versions of enzymes that lead to the formation of oncometabolites that may alter gene expression [9, 10]. Interestingly, not only can protein conformations be distinguished, but small antibody fragments of camelid origin (nanobodies) have even been reported to allow modification of the conformation of a target protein [11].

Although small molecule drugs can target the protein level directly, not all of the proteome is druggable by small molecule drugs. Not all proteins have active sites, and protein–protein interactions are more challenging to target with small molecules [12–15]. The large surface area via which antibodies can interact with different target shapes might explain the ease with which they allow interference with protein–protein interactions.

Given its advantages, how can the protein level be targeted? Antibodies can be expressed inside cells or delivered as proteins into cells. Approaches using antibodies are reviewed here with an emphasis on long-term proven robust approaches while discussing the strengths and challenges of individual methods. Applications in the research areas of neurobiology, viral infection and immunology or oncology are reviewed.

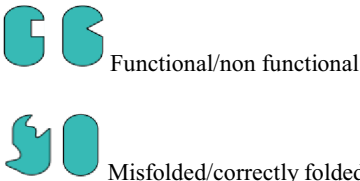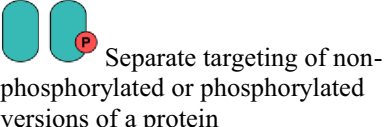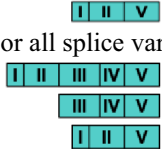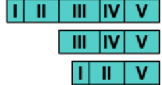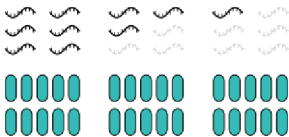## 2 Strategies to Use Antibodies Inside Living Cells

Antibodies can be used inside cells to neutralize targets, redirect proteins to different locations or simply analyse proteins of interest by tracking them. For instance, a membrane-tethered green fluorescent protein (GFP)-specific antibody was used to trap a GFP-fused morphogen at the cell surface, which abolished gradient formation [16]. This approach, termed morphotrap or nanotrap, demonstrates impressively how intracellular antibodies (intrabodies) can be used as tools to manipulate cellular processes by redirecting proteins of interest [16, 17]. In addition to interference, antibodies have also been used to trace and visualize proteins of interest in living cells [18, 19]. Tracing proteins not only qualitatively but also quantitatively with intrabodies allows studying protein turnover and the dynamic regulation of proteins [20]. Quantified visualization may require optimized intrabody expression [21]. Antibodies may furthermore be used to increase the turnover of a protein of interest (POI) via targeted proteolysis [6].

2.1 Sources for Binders

Molecules composed of amino acids that are able to specifically recognize another molecule are summarized under the general term 'binder' in the following. All binders can be used as intrabodies provided that the gene for the binder is available or the sequence is known. Depending on the compartment in which the intrabody is expressed, additional selection procedures might be required to ensure functionality of the binder in the chosen compartment [22, 23]. Methods to generate binders are briefly summarized in Table 2. For more detailed information, interested readers are referred to reviews on technologies for generating binders, such as display technologies including phage display, yeast display, ribosome display and bacterial display [24–29]. Among the group of recombinant binders, there are differences in species and in biochemical properties, which might be relevant for their use as intrabodies or other purposes. Binder species might not always be relevant if intrabodies are applied for research purposes, but therapeutic applications may require human or humanized antibodies. Certain species, such as sharks and camels, possess single-domain antibodies (sdAbs), a type of antibody that contains only a variable heavy chain (VH) but no light chain. Fragments of sdAbs of camelid origin, called nanobodies, are smaller in size than single-chain fragment variables (scFvs), a common antibody fragment of, for example, murine or human origin [30]. sdAbs may exhibit high thermal stability and the potential to refold [31, 32]. As an alternative to antibodies, designed synthetic scaffolds are potential sources for binders, such as designed ankyrin repeat proteins (DARPins), affibodies, fibronectin folds or alphaRep. Alternative scaffolds include synthetic molecules derived from ankyrin proteins (DARPins), fibronectin (e.g. monobodies), HEAT (huntingtin, elongation factor 3 [EF3], protein phosphatase 2A [PP2A], yeast kinase Tor1) protein-derived alphaRep or peptides derived from protein A (affibodies). In contrast to

Table 1 Targeting at the protein level: selected advantages

Distinguishable features	Examples for potential applications	References
Different functional conformations or pathological conformations	Functional/non functional Misfolded/correctly folded	GTPases in their functional or non-functional conformation (e.g., conformation-specific antibodies were generated for the GTPases Rab6 or dynamin [306–308]); misfolded vs correctly folded proteins
Post-translational modifications	Separate targeting of non-phosphorylated or phosphorylated versions of a protein	A phosphorylation site-specific intrabody targeting STAT3 was generated [270]
Splice variants	Targeting I II V only: I II V or all splice variants: I II III IV V III IV V I II V	Avoiding laborious multiple knockouts: instead of targeting individual isoforms, a family of proteins could be targeted with a single antibody instead of knocking out many genes, as suggested by Büssow and colleagues [50]
Different functional epitopes of the same protein	Specifically blocking the function of epitope 3 while preserving the functions of epitope 1 and 2	Functions of individual domains of cortactin were blocked by intracellular nanobodies: different outcomes when different epitopes of cortactin were targeted [275, 276]
Interfering with function in selected compartments	E.g., targeting exclusively the nucleus, exclusively the ER or exclusively the cytosol.	Functions can be blocked in defined cellular compartments as demonstrated by the knockdown of Sec61 by an intrabody [299]. Proteins with a similar sequence but residing in different cellular compartments can also be targeted individually at the protein level, using antibodies [253]
Functional interference independently of protein half-life	Knockdown at the RNA level relies on the turnover of a protein and proteins can often not be switched off faster than 48-72 h after treatment. Direct protein delivery to cells allows more rapid interference [6, 8] and allows acting on very long-lived proteins	Targets such as the protein Rec8 are normally hardly degraded and therefore difficult or impossible to deplete by methods acting on the DNA or RNA level, [8, 113]. Therefore, targeting at the protein level directly if applying targeted degradation allows interfering with function when other techniques fail

ER endoplasmic reticulum, *STAT3* signal transducer and activator of transcription 3

most natural antibodies, alternative scaffolds may not require the formation of disulfide bonds, so an additional selection procedure for functionality in the cytosol may be omitted [33–36]. The size and biochemical properties of binders may affect their suitability for intracellular use. Examples of binder formats that have already been used as intrabodies or for intracellular delivery are given in Table 2.

Table 2 Sources of binders and their suitability for use inside cells

Binder properties	Source for intrabody?	Source for protein delivery?	Methods to generate binders	Examples of binders	Use as an intrabody?	Use for protein delivery?
Polyclonal	–	+	Immunization	Full-length IgG	–	Clift et al. [8]
Monoclonal	–	+	Hybridoma technology	Full-length IgG	–	e.g. Clift et al. [8], Freund et al. [105], Desplacq et al. [317]
Recombinant	+	+	Gene isolated from a hybridoma, B cell clones or selected from libraries by display technologies	Full-length IgG	A construct of comparable size (150 kDa) containing constant domains of an IgG1 was reported as an ER intrabody [318] but not commonly used as an intrabody	e.g. Marschall et al. [3]
				Antibody fragments	Most commonly used type of intrabodies, e.g. scFvs or nanobodies [22, 91]	
				Alternative scaffolds	Examples for alternative scaffolds, e.g. DARPins, affibodies, monobodies, intracellular expression: Cetin et al. [319], Brauchle et al. [320], Vernet et al. [321]	

DARPins designed ankyrin repeat proteins, *ER* endoplasmic reticulum, *scFvs* single-chain fragment variables, – indicates no, + indicates yes

2.2 In Vivo Delivery of Binders or Binder Genes

Local in vivo electroporation has been reported for the delivery of proteins (antibodies) [37], but the in vivo delivery of proteins remains a challenge. The delivery of genes in vivo is much more advanced, with clinical trials for gene therapy in progress and some drugs already available, such as the US Food and Drug Administration (FDA)-approved gene therapy for spinal muscular atrophy (onasemnogene abeparvovec-xioi) or the in vivo gene therapy for treating an inherited eye disease (voretigene neparvovec). As a delivery vehicle for genes, adeno-associated viruses (AAVs) have been widely used, but gene therapy has also been performed with other virus types depending on the therapeutic goal, such as the FDA-approved genetically modified oncolytic virus for treating melanoma (talimogene laherparepvec) [38–43]. The therapeutic use of intrabodies is not limited to in vivo gene delivery, but intrabody genes could also be delivered ex vivo to blood cells, which could then be reinfused into the patient, as recently suggested by Png and colleagues [44]. Ex vivo gene delivery into T cells followed by reinfusion into patients has already been approved for treating B cell acute lymphoblastic leukaemia (tisagenlecleucel) [45] and large B cell lymphoma (axicabtagene ciloleucel) [46]. Ex vivo-transduced haematopoietic stem cells have

been approved for treating a genetic disorder that causes immunodeficiency (severe combined immunodeficiency due to adenosine deaminase deficiency [ADA-SCID]) [47]. Gene therapy is reviewed elsewhere in more depth [43, 48–50].

2.3 Compartments Conducive to Expression or Accessible to Externally Applied Antibodies

In nature, antibodies are produced in the secretory pathway, and recombinant expression in this compartment ensures optimal conditions for correct folding. Mitochondria have also been found to be conducive to correct folding of antibodies [22, 51]. Antibodies can be directed to various subcellular compartments using appropriate sorting signals [52, 53]. Intrabodies directed to the endoplasmic reticulum (ER) can be used to knock down membrane or secreted proteins by retaining these target proteins in the ER, if an ER retention signal is fused to the antibody.

The compartments that can be targeted easily by proteins include endosomes and lysosomes. This route of delivery has, for instance, been proven successful as part of the entry mechanism of antibody–drug conjugates (ADCs) [1, 54, 55]. The various routes for macromolecules into the cell are summarized in Fig. 1.

2.3.1 Membrane and Secreted Proteins: Knockdown with Endoplasmic Reticulum (ER) Intrabodies
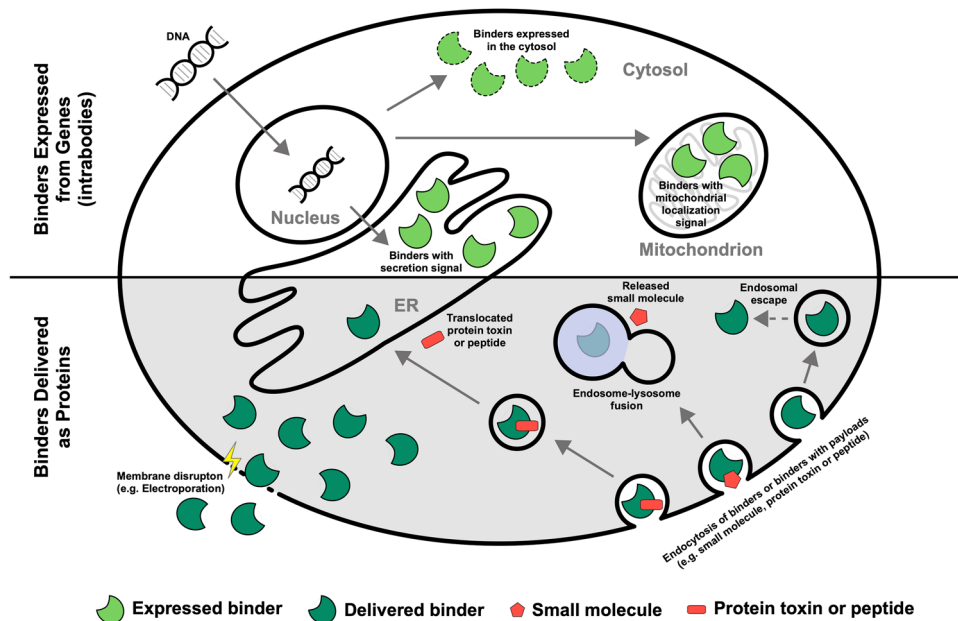
ER intrabodies are antibodies that are directed into the ER by a secretory leader peptide and retained in the ER by the ER retention motif KDEL. By binding to their target protein in the ER and retaining it there, ER intrabodies are able to keep their targets away from the place where they are functional. Knockdown depends on binding only, irrespective of which epitope is bound. Target proteins can be membrane proteins, such as the type I membrane protein vascular cell adhesion molecule 1 (VCAM1), which was knocked down by an ER intrabody in vitro and in vivo [56, 57]. Targets can also be secreted proteins such as interferon-α, the secretion of which was blocked by ER intrabodies in macrophages and dendritic cells [58]. The ER intrabody approach has already been successfully applied in many research areas. Examples of the application of ER-retained intrabodies with a focus on methodological and technological aspects have been reviewed previously [22], and their therapeutic potential has been briefly discussed [59, 60].

2.3.2 Mitochondria-Targeted Intrabodies

A target protein has been redirected away from its natural location using an antibody that was tethered to mitochondria using a mitochondrial outer membrane targeting element [61]. Antibodies containing a mitochondrial presequence, as described by Biocca and colleagues [51], correctly form intrachain disulfide bonds, in contrast to those expressed in the cytosol. Intrabodies have been used to analyse Ca^{2+} dynamics at the surface of mitochondria [62]. An antibody that was targeted to mitochondria and relocalized endogenous survivin to the intermembrane space of mitochondria was suggested as a tool to study mitochondrial protein import [63]. In another report, p53 was immobilized at the mitochondrial outer membrane, resulting in reduced cell viability and morphological changes of mitochondria [64].

2.3.3 Antibody Delivery Routes: Endosomes, Lysosomes, ER-Associated Degradation (ERAD) and How to Use It

Delivery of proteins to endosomes and lysosomes can be easily and efficiently achieved, even if proteins often cannot escape from endosomes [65, 66]. How can antibodies be employed for therapy in these compartments? One example of the use of this cell entry route is ADCs, which consist

Fig. 1 Routes into the cell for antibodies. Antibodies can be expressed in cells or delivered as proteins to cells. Antibodies fold correctly in the endoplasmic reticulum (ER), where they are naturally expressed, as well as in mitochondria. The expression of antibodies in the cytosol may require selection for antibodies that are suitable for folding in the cytosol (dashed lines) using one of the cytosolic intrabody selection technologies. Antibodies can be delivered as proteins to the cytosol, for instance, by physical methods that are associated with membrane disruption. The critical step for antibodies that are delivered via a route that involves endosomal uptake is overcoming endosomal entrapment, which has been a major efficiency bottleneck for some of those approaches. The delivery of antibodies to the lysosome can serve to deliver small molecules to the cytosol of selected cell types, such as in antibody–drug conjugates (ADCs). Small protein toxins or peptides have been delivered to the cytosol via retro-translocation from the ER to the cytosol

of antibodies linked to a small molecule, such as a chemotherapeutic, kinase inhibitor or antibiotic [67–69]. While the antibody cannot cross the membrane, small molecules can, if freed from the antibody via a cleavable linker or by degradation of the antibody in the lysosome [70–72]. The proven success of ADCs in clinical practice (e.g. an ADC that was approved for the therapy of metastatic breast cancer in 2013) suggests that this route of delivery is effective.

A different delivery route is exemplified by immunotoxins, which consist of an antibody linked to a toxin, often a protein of bacterial origin [73]. Similar to ADCs, the toxin has to be released from the antibody, which can be achieved by a cleavage site [74, 75]. After release, small proteins, such as bacterial toxins can, for instance, reach the cytosol via the ER-associated degradation (ERAD) pathway [76]. Proteins are normally degraded as part of the ERAD. A low lysine content in the toxin ricin is assumed to promote the escape of some toxin molecules from degradation, which is sufficient for the toxin to be effective [77–79]. An immunotoxin for the treatment of leukaemia was approved for therapy in 2018 [55, 80]. This route of delivery is suitable for all proteins that readily refold and are effective at the dose of proteins that can escape degradation during ERAD.

The refolding of molecules translocated to the cytosol via ERAD is not necessary if the molecule is a peptide. A construct containing a peptide that can regulate immune reactions if delivered to the cytosol showed promising results in mouse models for rheumatoid arthritis [81, 82].

2.4 Compartments with No Natural Expression or Inaccessible to Externally Applied Antibodies

2.4.1 Cytosolic and Nuclear Intrabodies

Antibodies in the secretory compartment fold correctly, and antibodies form disulfide bonds in mitochondria. In contrast, antibodies do not form disulfide bonds in the cytosol [51], and only a few antibodies (estimated to be as low as 1% of antibodies) can be expressed as high-quality molecules in this compartment [23, 83, 84]. Approximately 0.1% of antibodies from a naive human spleen cell-derived scFv library were stable and functional [83]. Engineering a net charge was proposed as a strategy for generating antibodies that do not aggregate in the cytosol [85]. Certain frameworks may increase the chance of expression as functional antibodies in the cytosol [86–89]. Such frameworks may be selected antibody scaffolds that are functional even in the absence of disulfide bonds [90] or non-antibody scaffolds [35, 36]. The properties of nanobodies render them more suitable for expression in the cytosol [91], but various tendencies for aggregation may still occur [92, 93]. Non-antibody scaffolds may also not always be functional; only 10–20% of a fibronectin-derived alternative scaffold called FingR

(Fibronectin intrabody generated with messenger RNA display) that was target-specific in vitro also co-localized with its target intracellularly [94]. Therefore, technologies such as intracellular antibody capture technology (IACT), intrabody selection after tat export (ISELATE) and "quality control" were developed to select antibodies that are suitable for cytosolic expression, which are reviewed in Marschall et al. [22, 23] and described in more detail elsewhere [83, 89, 95]. Additionally, a bacterial two-hybrid system was developed as a selection method for nanobodies as intrabodies, which has the advantage of higher transformation efficiencies and thus the chance for obtaining higher diversity than yeast two-hybrid methods [96]. To eliminate self-oligomerizing intrabodies from an intrabody library, a Fas-associated death domain (FADD) was fused to antibodies as a suicide switch against unwanted intrabodies [97]. Antibodies that are functional if expressed in the cytosol can also be directed to the nucleus if provided with the respective signal peptides. In contrast to ER intrabodies, cytosolic intrabodies need to bind a particular epitope on the target to neutralize its function [22]. Both the requirement for selecting antibodies that can fold correctly in the cytosol and the requirement for neutralizing properties make cytosolic intrabodies laborious to generate. However, cytosolic intrabodies allow promising applications, such as a recently described concept using two intrabodies against a target to sense and trigger a response to this target [98].

2.4.2 Protein Delivery to the Cytosol or Nucleus

A plethora of methods for the delivery of proteins have been suggested, with varying degrees of success and validation. Because one plasmid can express many proteins, low delivery efficiencies are less critical for DNA than for most proteins. A few enzymes may convert many substrate molecules, but antibodies usually have to be delivered in excess to intracellular molecules to cause an effect. Past results have demonstrated the crucial importance of choosing appropriate methods for evaluating the cytosolic delivery of proteins to avoid artefacts and the overestimation of delivery efficiency [2–4, 99].

Methods for protein delivery include approaches based on physical membrane disruption and approaches that rely on the endocytotic uptake of cargo with subsequent endosomal release [100]. For the latter class, endosomal entrapment has in the past been found to pose a major block to delivery efficiency [101, 102]. Further delivery methods include approaches that combine physical methods with chemicals, such as the application of graphene quantum dots to cells and laser irradiation [103], or use microorganisms to deliver cargoes [104]. Among the many proposed approaches to the cytosolic delivery of proteins, disruption of the membrane has proven particularly efficient for the delivery of proteins

into cells [3, 105, 106]. With an efficiency of up to 90–99%, high cell viability of 80–90% [107, 108] and validation in many cell types over the last 20 years (Table 3), electroporation is a robust approach for delivering antibodies into the cytosol. Further physical methods include microinjection or microfluidic cell squeezing, which are either applicable to lower cell numbers or require special equipment [106]. Delivered antibodies remain functional in the cytosol in spite of the reducing environment. The half-life of antibodies in the cytosol is long and they bind to their targets even 3–4 days after electroporation [3, 105]. This suggests that degradation might be even slower than dilution of the antibody by cell division. Compared with DNA that has integrated into the genome, protein delivery is not permanent but transient and depends on the half-life of the delivered protein. The time-limited activity of a protein drug offers the opportunity for better control over therapy and thus higher safety.

3 Targeted Protein Degradation

Targeted proteolysis as an alternative to RNA interference (RNAi) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9-based knockdown allows rapid interference with protein levels, which is independent of regulation on the RNA and DNA level. While tools for performing knockdown via RNAi or CRISPR/Cas9 have so far been accessible more easily, the field of targeted protein degradation has recently undergone rapid progress. Targeted proteolysis can be mediated both by intrabodies (antibodies expressed intracellularly) and delivered antibodies (protein delivery). Two types of targeted proteolysis are distinguished here: approaches using degrons and approaches using high-affinity binders (Table 4).

3.1 Targeted Proteolysis by Modifying the Protein of Interest (POI)

Degrons enable proteolysis by fusing a short sequence to the POI, which means that the POI needs to be modified. Proteolysis approaches based on high-affinity binders may use macromolecules or small molecules as binders and can act on endogenous, unmodified POIs. The proteolysis of degron-tagged proteins can be controlled by adding a small molecule that either induces degradation upon addition or induces degradation upon withdrawal. The auxin-inducible degron (AiD), a proteolysis approach based on a plant hormone, allows degradation of the degron-tagged POI upon the addition of a small molecule. Upon fusing a POI with a SMASh (small molecule-assisted shutoff)-tag, the SMASh-tagged POI can also be degraded upon addition of a small molecule, but because of its special property of promoting the proteolysis of newly synthesized proteins upon addition of the chemical, this degron allows research on the protein half-life and degradation of the POI. In contrast, for FKBP12 (12 kDa FK506-binding protein)- or UnaG-tag based degrons the degron-tagged POI is stabilized upon addition of the small molecule and degraded as soon as the small molecule is absent [5, 6, 109–111].

3.2 Targeted Proteolysis Via High-Affinity Binders: Keeping the POI Unmodified

High-affinity binders allow the targeted proteolysis of unmodified POIs. Proteolysis-targeting chimaeras (PRO-TACs), which are bispecific binders, have recently gained much attention, with the first one reaching clinical studies [112, 113]. The concept of bispecificity is well-known for intercellular applications, such as bispecific antibodies that link tumour cells and immune cells to promote the killing of tumour cells [114]. The same concept can also be used for intracellular applications. By recruiting the cellular degradation machinery to a POI, degradation of the chosen protein can be achieved. The first PROTACs, which were based on peptides, suffered from a lack of cell permeability. More recent generations of PROTACs were constructed from small molecules and have shown improved cell permeability [113, 115]. Because PROTACs keep recruiting POIs, a single PROTAC molecule can induce the proteolysis of many POI molecules [116]. PROTACs have already been used successfully in vivo, and an oral PROTAC drug has been approved for a phase I clinical trial as a drug for the treatment of prostate cancer. In spite of the great success of PROTACs, future work is still needed; for instance, Guo and colleagues [117] state that there is still a "challenge for designing on-target PROTACs" [118]. Although small-molecule PROTACs are cell permeable, in contrast to antibodies, the high specificity and almost unlimited diversity of proteins that can be targeted by antibodies make them an attractive tool for targeted degradation. More than 30 proteins have already been targeted by PROTACs [115], but hundreds of antigens can already be targeted via antibodies derived from phage display with HAL libraries alone [24], not including the enormous number of antibodies from other sources allowing access to the antibody gene. The technology for generating antibodies specific for a new POI is a well-established and robust procedure [24]. The high number of proteins that can be targeted with antibodies can therefore justify the trade-off between diversity of targeted antigens and deliverability that is more favourable in small-molecule PROTACs.

Antibodies can, for instance, be delivered into cells via electroporation or microinjection. Delivering full-length IgGs to cells has been described as a means to achieve the degradation of a POI dependent on the tripartite motif

Table 3 Antibodies or proteins delivered by electroporation

Delivered protein	Cell type	References
Asparagine synthetase antibody	HeLa, HT-5, and L5178Y DlO/R	[107]
p21^{ras} antibody	B16BL6 mouse melanoma cells	[322]
Various antibodies	Human cells	[323]
MLCK antibody, constitutively active form of MLCK	Macrophages	[324]
Various antibodies and proteins	Pheochromocytoma, other cultured cells	[325]
p21^{ras} antibody	B16BL6 mouse melanoma cells	[326]
Tubulin antibody	CHO cells	[327]
TK enzyme	TK-deficient mammalian cell line	[328]
Lipoxygenase antibody	Lentil protoplasts	[329]
Vimentin antibody, RNAse A	Fibroblasts	[330]
Lipoxygenase antibody	Lentil protoplasts	[331]
Cyclin D1 antibody	Mouse embryo and SKUT1B cells	[332]
TGN38-, p200- and VSV-G antibodies	NRK-6G cells	[333]
Tropomodulin, antibodies specific to: tropomodulin, talin, vinculin and a-actinin	Fibroblasts	[334]
Lucifer yellow, IgG	SW 3T3, NIH 3T3, dHL60, A7r5, BASM	[335]
MAP kinase antibody	MDCK cells	[336]
Anti-M-line protein, titin antibodies	Chicken cardiomyocytes	[337]
Various antibodies	Chicken cardiomyocytes	[338]
Wild-type STAT1, mutated STAT1, STAT1 antibody	Rat mesangial cells	[339]
DNase I, restriction enzymes	Jurkat cells	[340]
c-Src antibody	Human vascular smooth muscle cells	[341]
TFAR19 antibody	HeLa	[342]
c-Fos antibody	Spinal neuronal cells	[37]
STIM1 antibody	Platelets	[343]
Orai1 antibody	Platelets	[344]
EGFP	HeLa	[345]
Orai1 antibody	Platelets	[346]
HPV16 E6 oncoprotein, PCNA, RNA polymerase II largest subunit	HeLa, CaSki, H1299, MEL501 and U2OS	[105]
Fc-Cre, tubulin antibody, myosin antibody	SC1 REW22, HeLa	[3]
PCNA, DNA polymerase alpha	HeLa, US2-OS	[317]
Pericentrin, mTOR, IκBα, NLRP3, anti-IKKα antibodies	NIH3T3, HEK293T, human monocyte-derived macrophages	[8]
RBP1, TBP and TAF10 antibodies	Various mammalian or *Drosophila melanogaster* cell types	[108]
γ H2AX antibody	U2-OS	[347]
Various recombinant proteins	Various cell lines	[348]

BASM bovine aortic smooth muscle, *CHO* Chinese hamster ovary, c-*Src* cellular Src, *EGFP* enhanced green fluorescent protein, *H2AX* H2A.X variant histone, *HPV16* human papillomavirus 16, *IKKα* IκB kinase α, *MAP* mitogen-activated protein, *MDCK* Madin-Darby canine kidney, *MLCK* myosin light chain kinase, *mTOR* mammalian target of rapamycin, *NLRP3* NLR family pyrin domain containing 3, *NRK* normal rat kidney, *Orai1* ORAI calcium release-activated calcium modulator 1, *PCNA* proliferating cell nuclear antigen, *RBP1* retinol binding protein 1, *STAT* signal transducer and activator of transcription, *STIM1* stromal interaction molecule 1, *TAF10* TATA-box binding protein associated factor 10, *TBP* TATA box binding protein, *TFAR19* TF-1 apoptosis-related gene 19, *TK* thymidine kinase

protein TRIM21 (tripartite motif containing-21)/Ro52 [8]. TRIM21/Ro52 first attracted attention due to its particular antigenicity and as a target for autoantibodies in autoimmune diseases [119]. TRIM21/Ro52 is an E3 ubiquitin ligase that has been proposed to act as a mechanism of intracellular antiviral defence by binding to the Fc part of antibodies attached to viruses that enter the cytosol and subsequently induce degradation of antibodies and viruses via

the proteasome [120]. Targeted degradation via TRIM21 can therefore be achieved using full-length antibodies or by using proteins fused to the Fc part of an antibody. While all cells can express TRIM21, it is expressed at different levels in different cell types [8]. The efficiency of targeted degradation depends on the expression level of TRIM21 as well as the amount of antibody and the amount of target present. TRIM21-mediated targeted proteolysis was reported to be

Table 4 Comparison of strategies for targeted degradation

Type	Module to target for degradation	Requirements and features
POI fusion	Degron	Modification of the POI and genetic modification of the cell/organism required
High-affinity binder based (unmodified POI)	TRIM21	Binder with a constant (Fc) domain has to be used, protein delivery required
	Bispecific small molecule (PROTAC)	Availability of a small molecule binder for the POI required
	Binder fused to a degron	Either expression of the binder from a gene or protein delivery can be used; no Fc domain is required, and therefore more choice is available for the binder format/type (e.g. IgG, scFv, nanobody, alternative scaffolds)

POI protein of interest, *PROTAC* proteolysis-targeting chimaera, *scFv* single-chain fragment variable, *TRIM21* tripartite motif containing-21

rapid, occurring within minutes [8]. Although endogenous TRIM21 levels may be sufficient in some cells, TRIM21 has to be overexpressed or delivered as a protein to mediate proteolysis sufficiently in other cells [8]. Using antibody- and TRIM21-mediated proteolysis, a method termed 'Trim away', allowed depletion of the long-lived protein Rec8, which has a role in sister chromatid cohesion and does not turn over for long time periods in mice and possibly for years in humans. If there is a lack of turnover of the POI, depletion via RNAi would be ineffective. Gene knockout is furthermore not possible if a gene is essential for viability [8, 121, 122]. Furthermore, by using antibodies that specifically recognize a pathogenic version of the protein huntingtin, Trim away allowed selective depletion of only the pathogenic protein variant that was co-expressed with the normal huntingtin protein [8]. This demonstrates the possibility of selectively depleting protein variants, which could also be employed for selectively depleting proteins with post-translational modifications.

Targeted degradation was also mediated by expressing antibodies in cells instead of delivering them as proteins. The binder is genetically engineered for targeted proteolysis, leaving the POI unmodified [6]. Selecting antibodies that are functional if expressed in the cytosol is often more elaborate than normal antibody generation procedures, but the use of an anti-GFP antibody allowed a single antibody to target many antigens for degradation if GFP-tagged [123]. This approach is especially attractive for model organisms with GFP-tagged proteins, such as those often used in developmental biology. In zebrafish and flies, this approach has already been successfully applied [124–126]. Various tags and motifs can be employed to target antibodies and their antigens for degradation, such as providing the binder with an AiD, a proline, aspartate or glutamate, serine and threonine (PEST) motif, the Von Hippel Lindau protein, Speckle-type POZ protein (SPOP), an F-box domain or the catalytic domain of a ubiquitin ligase [6, 124, 127–132].

A nanobody fused to an AiD allowed inactivation of the anaphase-promoting complex/cyclosome (APC/C) in vitro and in zebrafish. Genetic engineering was required to allow expression of the nanobody–degron fusion, and this approach also required expression of the plant F-box protein, protein transport inhibitor response 1 (TIR1). The advantage of this approach was its reversibility as well as rapid and effective temporal control over degradation via auxin supply. Marked degradation occurred within 16–28 min, and protein levels had recovered sufficiently to rescue the knockdown phenotype 8 h after the removal of auxin [131]. An intrabody fused to an F-box domain was used to specifically degrade the active form of the GTPase RHOB [132]. 'Ubiquibodies' are ubiquitin ligases in which the natural substrate binding domain has been replaced by an antibody, which allows the targeting of POIs for degradation [127]. Integrating PEST motifs into the sequence of a binder has also been used for the purpose of targeted degradation [130, 133, 134], so, overall, there is a wide range of strategies and tools available to target proteins for degradation once an antibody can access a POI.

3.3 Targeted Proteolysis Via Lysosomes or the ER

The approaches mentioned so far employed the cytosolic degradation machinery, but approaches to perform targeted degradation in other compartments have also been described. Lysosome-targeting chimaeras (LYTACs) are based on the strategy of employing the cation-independent mannose-6-phosphate receptor (CI-M6PR) to target POIs for lysosomal degradation. LYTACs consist of an antibody that is fused to a ligand for this receptor and allows targeting membrane proteins and secreted proteins for lysosomal degradation [135]. Further strategies for lysosomal targeting include approaches based on receptor crosslinking and targeting to the lysosome by means of peptides [65, 66]. Degradins allow targeting of proteins in the ER for degradation. Degradins consist of a moiety that is specific for the POI and the protein SEL1L, which is involved in identifying misfolded proteins in the ER to subject them to the ERAD pathway. Degradins were thus proposed as a strategy to retrotranslocate POIs from the ER to the cytosol for proteasomal degradation [136].

4 Applications in Neurobiology

Many neurodegenerative diseases are associated with protein misfolding and aggregation [137]. There is controversy about which forms of proteins are toxic in neurodegenerative disorders. While some studies attribute toxicity to the aggregated form, other studies suggest aggregation to be a beneficial process that protects the cell from intermediate or misfolded toxic proteins [138, 139]. Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are also discussed as potential prion-like diseases for which misfolded proteins are known to propagate and convert other proteins into pathological forms [140, 141]. Because protein aggregation occurs downstream of the gene level, studying neurodegenerative diseases requires methods that allow direct targeting of proteins. To date, there is no cure for neurodegenerative diseases, and all available treatments only manage symptoms or halt disease progression. Drug repurposing, which is the evaluation of existing drugs normally used to treat other diseases such as cancer, asthma, infections and others for their therapeutic potential in treating neurodegenerative diseases, is an active area of research with the advantage of already pre-existing knowledge about the pharmacokinetic and pharmacodynamic profiles of drugs [142]. Beyond repurposing existing drugs, further research on molecular mechanisms and potential new therapies for neurodegenerative diseases is desirable. With their suitability for targeting different conformations and aberrant versions of protein structures, antibodies are particularly interesting as research tools or as potential new therapies for protein misfolding diseases (Table 5).

4.1 Parkinson's Disease

PD, the second most common neurodegenerative disease, is characterized by the loss of dopaminergic neurons, and is best known for causing motor symptoms. PD is associated with misfolded forms of the protein α-synuclein, which therefore has been considered of interest as a potential drug target [143]. Several α-synuclein intrabodies and engineered versions thereof have been described to reduce aggregation and toxicity in vitro [144–148]. In vivo, gene therapy using AAV-based vectors has been performed to deliver α-synuclein intrabodies into rats. Because antibodies can be employed to target selected regions of a protein, intrabodies can be used to map structure function relationships in detail. This advantage was used by employing two different intrabodies, targeting the non-amyloid or the C-terminal part of α-synuclein. The two different intrabodies were further engineered with a PEST motif for proteasomal degradation. One intrabody showed marked maintenance of striatal dopaminergic tone compared with controls. Furthermore, modest behavioural rescue was observed, although pronounced variability occurred among individual animals. While authors mention the importance of evaluating the model system as well as the timeline of therapeutic intervention, this study demonstrates the feasibility and potential of intrabody gene therapy in vivo [130].

4.2 Alzheimer's Disease

Gene therapy to express antibodies in animal models has also been applied multiple times as a potential new treatment for AD and was reviewed in depth by Cardinale et al. [149] and Meli et al. [150]. AD is the most common neurodegenerative disease causing dementia. It is associated with the aggregation of amyloid-β (Aβ) peptide, which therefore raised interest as a potential drug target [151]. The Aβ peptide is formed via proteolysis of the Aβ precursor protein (APP), and intrabody binding to an epitope adjacent to the cleavage site reduces Aβ formation by shielding the cleavage site. Another even more efficient intrabody almost completely prevented Aβ formation by inducing disposal of APP from the ER [152]. As a complementary approach, an intrabody was used to suppress the function of a protein involved in APP proteolysis [153]. Instead of targeting cleavage, the different products of cleavage can also selectively be distinguished by intrabodies. Misfolding leads to the formation of pathological aggregates, as has been demonstrated using conformation-specific intrabodies. This opens up promising new therapeutic avenues, since conformation-specific intrabodies could target only those Aβ conformers that undergo pathological oligomerization without interfering with the 'healthy' processing of APP [154]. The intrabody could thus act as a filter that allows only 'healthy' conformers to pass. Gene therapy via the AAV-mediated delivery of an anti-Aβ42 intrabody in an AD mouse model allowed partial clearance of Aβ42 deposits [155], and in a follow-up study, myelin integrity was restored in mice treated with intrabody gene therapy [156]. An intrabody was furthermore used for imaging purposes in live neurons to study mechanisms of memory impairment in AD [157], and intrabodies have been found to be generally valuable tools for imaging purposes in neurons [158].

AD is also associated with abnormal tau phosphorylation as well as aggregation and is the most common tauopathy [159, 160]. Melchionna and Cattaneo [161] reported the degradation of tau by intrabody-mediated tumour necrosis factor (TNF)-α inducible targeted proteolysis. However, this approach might be limited as a therapeutic approach due to the adverse effects of the cytokine TNF-α and is more likely to serve as a research tool [162], as reviewed by Messer and Butler [160]. Targeted degradation of tau via expressing an AAV-delivered intrabody resulted in a statistically significant decrease in tau-associated pathology in a human-tau

Table 5 Intracellular antibodies applied in research and as potential therapeutic strategies

Research area	Disease/disease process	References
Neurobiology	Alzheimer's disease	[152–157, 161, 163, 165, 349]
	Parkinson's disease	[130, 136, 145–148]
	Huntington's disease	[133, 168, 170–175, 310, 350, 351]
	Amyotrophic lateral sclerosis	[182, 184]
	OPMD	[185]
Viral infections	HIV	[213, 215, 217, 218, 221, 222, 224–227, 229]
	EBV (HHV-4)	[235, 236]
	HHV-8	[238, 241]
	HBV	[259–261, 352]÷
	HCV	[265–267, 270, 353]
	HPV	[246–251]
	Rotavirus	[199]
	Influenza A	[202, 203]
	Ebola	[209, 354]
	Marburg virus	[209]
	Bluetongue virus	[355]
	Hantavirus	[356]
	Vesicular stomatitis virus	[357]
	Rabies	[358]
	Porcine viruses	[359, 360]
	Maedi visna virus	[361]
	Flavivirus	[362]
	Cucumber mosaic virus	[194, 196]
Cancer research	Cellular level: oncogenic pathways, proliferation, cell cycle, apoptosis	[64, 274, 276, 278, 363, 356, 364–369]
	Tissue level: adhesion, metastasis, angiogenesis	[132, 279–288, 370–377]
	Neo-antigens	[291, 295, 296]

EBV Epstein-Barr virus, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *HHV* human herpesvirus, *HPV* human papillomavirus, *OPMD* oculopharyngeal muscular dystrophy

transgenic mouse model as reported by Gallardo and colleagues [163]. Among several splice variants of tau, the 2 N isoform has been suggested to preferentially interact with proteins that are associated with neurodegenerative diseases [164]. The effect on a selected isoform could be imagined to be more effective if targeted by an antibody specific for only an individual splice variant compared with a drug that recognizes all isoforms because binding does not compete with the other isoforms. A combination of focused ultrasound and administration of an antibody specific for the 2 N isoform of tau was applied to deliver the antibody to the brains of mice. This treatment was found to reduce tau hyperphosphorylation and anxiety-like behaviour in a transgenic mouse model that overexpresses the 2 N isoform of tau [165].

4.3 Huntington's Disease

Huntington's disease (HD) is a hereditary disorder caused by a mutant version of the huntingtin gene (*mHtt*), which is associated with motor symptoms and cognitive decline as a consequence of the death of brain cells in the striatum [166]. If gene therapy is used to target *mHtt* as a treatment for HD, the pathologic protein could still remain in the body and cause pathologic effects due to its ability to act as a seed and convert other proteins into pathologic molecules [167]. For this reason, even in the absence of the mutated gene, the pathological protein would have to be removed, which could be performed with intrabodies. Multiple studies have already focused on intrabodies as a promising treatment option for HD, which are reviewed in detail by Denis et al. [168, 169]. In vitro, intrabody expression resulted in decreased *mHtt* aggregates or increased *mHtt* turnover and increased cell survival. Therapeutic effects were enhanced when drug administration was combined with intrabody expression in vivo in a fly model [170]. In transgenic mice or mice that received intrabody gene therapy, decreased motor and cognitive impairments and increased body weight and lifespan were observed [169, 171–175].

4.4 Further Applications in Neurobiology

Amyotrophic lateral sclerosis (AML) is a neurodegenerative disease characterized by motor deficits, which can also be associated with cognitive and behavioural changes. In most cases the mechanisms causing the disease are unknown, called sporadic ALS, but in a subset of patients the disease is familial involving mutations in the genes *C9orf72*, *TARDBP* (TDP43), *FUS* and superoxide dismutase (*SOD1*) among others [176–180]. One hypothesis is that the degeneration of motor neurons in AML caused by mutated versions of *SOD1*, might be associated with the tendency of mutant *SOD1* versions to misfold and aggregate [176]. Ghadge and colleagues [181] generated two anti-*SOD1* antibodies, which prolonged survival in a mouse model after delivery by gene therapy. Gene therapy was also performed to express antibodies specific for misfolded *SOD1* in mice, resulting in delayed onset of ALS pathogenesis and an extended lifespan [182]. TDP43 is an RNA/DNA binding protein involved in the regulation of gene expression and RNA processing. TDP43 aggregates not only in ALS but in other neurodegenerative diseases such as frontotemporal lobar degeneration (FTLD), therefore being a promising target for multiple diseases [183]. An antibody specific to a selected domain of TDP43, RNA recognition motif 1 (RRM1), was generated by Pozzi and colleagues [184] to obtain two therapeutic effects at the same time. The TDP43-specific antibody should interfere with protein aggregation and additionally combat hyperactive inflammatory responses by blocking protein–protein interactions of the RRM1 domain. Gene therapy of mice with this antibody had a protective effect and could be a novel therapeutic avenue for AML and for TDP43 tauopathies in general [184].

Oculopharyngeal muscular dystrophy (OPMD), a protein aggregation disease that is characterized by the weakening of specific muscle groups involved in holding the eyelids and swallowing and of proximal limb muscles, was nearly completely rescued in vivo by an intrabody in a fly model [185].

Apoptosis might be responsible for cell death in several neurodegenerative diseases. Inhibition of the proapoptotic protein Bax was proposed as a means to confer resistance of cells against apoptosis [186].

The optimization of intrabodies to treat neurodegenerative disorders was reviewed in depth in a recent publication by Messer and Butler [160].

5 Applications in Viral Infections

Intrabodies used in the research area of viral infections belong to the class of cytosolic intrabodies or the class of ER intrabodies, if the proteins of interest are in the secretory pathway [58, 187–193]. The intracellular use of antibodies has been considered a valuable option by researchers for targeting many viruses (Table 5). The ease with which plants can be genetically modified allows conferring an antibody-based 'immune response' on plants. Intrabodies were expressed in tobacco and tomato plants as a means of protecting them from infection with the cucumber mosaic virus, which has economic relevance due to its wide host range and worldwide occurrence [194–196]. Antibodies from camelid origin have also been reported to be expressed in plants [197, 198].

Understanding the mechanistic details of infections through intrabody-mediated interference may help with identifying new strategies for pharmaceutical intervention. An advantage of applying intrabodies in viral infection research is the possibility of disrupting viral protein function without genetic manipulation of the virus itself. For instance, an intrabody was used to study rotavirus [199], a non-enveloped RNA virus causing gastroenteritis [200]. Intrabodies also allowed the mechanistic details of influenza A, an enveloped RNA virus, to be studied [201–203]. Because genetic engineering often impairs or abrogates viral function for small RNA viruses such as influenza A [202, 204–206] and inhibition by chemicals is not always possible, intrabodies allow questions to be answered that cannot be addressed by these methods. Beyond analysing virus biology, intrabodies were also used in a more therapy-oriented approach. Infection with Ebola and Marburg virus, which are both enveloped RNA filoviruses [207], poses a significant threat due to their high lethality rates of up to 90% in some cases of outbreaks [208]. As a strategy to allow only empty and non-infectious particles to form by inhibiting packaging, viral proteins from Ebola and Marburg virus were crosslinked inside the cell via a dimeric intrabody [209].

5.1 Chronic Viral Infections and Oncoviruses

5.1.1 Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is an enveloped (+)-strand RNA retrovirus that can eventually cause acquired immunodeficiency syndrome (AIDS), causing distinct depletion of CD4+ T cells and thus death by progressive failure of the immune system if not treated. A combination of antiretroviral drugs is used to manage the disease as a chronic condition to prevent the development of AIDS in patients [210–212]. Similar to the approach of combination therapies used in HIV treatment, intrabodies were used to target different points in the infectious cycle. Both viral and host proteins have been targeted by intrabodies to attack HIV, including the matrix protein p17 [213], the viral surface protein gp120, which is responsible for binding to the cellular receptor [214, 215], gp41, which is involved in fusion of virus and host membranes [216, 217],

the virion infectivity factor Vif, which counteracts antiviral mechanisms of the host [218, 219], Rev, which is responsible for the export of viral RNA from the nucleus [220, 221], the viral accessory protein Vpr, which disturbs cellular pathways [222, 223], the viral protein TAT, which allows viral gene expression [215, 224–226], the cellular protein LEDGF (lens epithelium-derived growth factor) [227], and cellular receptors involved in viral entry of HIV into cells, such as the host proteins CCR5 (C–C chemokine receptor type 5) [189, 191, 193] and C-X-C chemokine receptor type 4 (CXCR4) [190, 192, 228]. An intrabody fused to a fluorescent protein was further used to allow dynamic tracing of HIV in cells [229]. To interrupt viral replication by allowing only non-infective virions to form, intrabodies were expressed that were targeted for degradation and specific for gp160 [230]. Because of its high mutation rate, HIV continues to change in patients, which can result in drug resistance [231]. A way to circumvent this problem could be targeting host proteins that are relevant in the viral life cycle, but do not have high underlying mutation rates like the viral proteins. Protein–protein interaction between a viral and a host protein was blocked by an intrabody as a means to interfere with viral replication and proposed as a potential intracellular immunization for T cells in HIV-positive patients [227]. By expressing an ER intrabody that retained the host cell surface receptor CCR5 in the ER of CD4+ T cells, these cells were protected from viral entry both in vitro and in a mouse model [193]. Because this approach is also based on targeting a host protein, the ER intrabody is not at risk of losing the ability to bind its target, as might occur with escape mutants if a viral protein is targeted. To address problems with drug resistance and residual infected reservoir populations under antiretroviral therapy, alternative approaches such as gene therapy could be further explored to improve therapeutic efficacy. Viral gene expression is stimulated by the HIV-1 Tat protein [232]. In a preclinical study with rhesus macaques, CD4+ T cells were isolated from macaques for ex vivo transduction with an HIV-1-Tat-specific intrabody to promote the survival of CD4+ T cells. Modified CD4+ T cells were re-infused into macaques, and the animals were subsequently challenged with the virus. CD4+ T cells expressing the Tat-specific intrabody survived longer than cells expressing a control intrabody after challenge, and a reduced viral load was observed in one of two animals [226].

5.1.2 Epstein–Barr Virus

Epstein–Barr virus (EBV), also called human herpesvirus 4 (HHV-4), is a DNA virus that may cause cell transformation and has a global prevalence of more than 90%. Although EBV infection is in most cases lifelong dormant, EBV was the first virus identified as an oncovirus and is associated with cancers such as lymphomas and epithelial cancer and is a risk factor after transplantation for developing post-transplant lymphoproliferative disease (PTLD) [233, 234]. Latent membrane protein 1 (LMP1) of EBV is essential for B cell transformation, and its knockdown was achieved by expressing an ER intrabody. By reducing LMP1 levels via an ER intrabody, cells were rendered more sensitive to chemotherapeutic-induced cell death [235]. The same target, LMP1, was also attacked at the cytosolic part of the LMP1 transmembrane protein by a cytosolic intrabody. To induce a functional effect with a cytosolic intrabody, an antibody was generated against a selected epitope of LMP1, which serves as a docking site for downstream signalling molecules [236]. This demonstrates how the same protein can be targeted in different ways by intrabodies and how the experimental approach differs accordingly. As a therapeutic strategy, EBV-infected tumour cells could be sensitized to chemotherapeutic drugs by ER intrabodies or by cytosolic intrabodies as gene medicines [235, 236].

5.1.3 Human Herpesvirus 8

Kaposi sarcoma-associated herpesvirus, also called human herpesvirus 8 (HHV-8), has a global prevalence of less than 10% but a regional prevalence of up to 70%. HHV-8 is an enveloped DNA virus that is usually asymptomatic upon primary infection but can cause Kaposi sarcoma in immuno-deficient individuals [234]. Latency-associated nuclear antigen (LANA1) helps to maintain the viral DNA of HHV-8 in daughter cells [237] and was therefore chosen as a promising drug target for intracellularly expressed antibodies [238]. A cancer-promoting feature of HHV-8 is its expression of proteins that are very similar to host proteins and it can therefore interfere with regulation of the host's cell cycle, apoptosis and cytokine signalling. Viral interleukin-6 (vIL6) mimics the activities of human interleukin-6 (hIL6), but stimulation by hIL6 depends on hIL6 receptor (hIL6R), while vIL6 can stimulate cells even independently of hIL6R [239, 240]. By the expression of an ER intrabody that retained vIL6 in the ER, the secretion of vIL6 was prevented, and vIL6-mediated signalling was blocked [241].

5.1.4 Human Papillomavirus

Human papillomavirus (HPV) has a prevalence of 70 million cases in the USA alone. Each year, an estimated 500,000 cases of cervical cancer occur, which are caused by high-risk HPV strains. The first prophylactic vaccine against high-risk HPV strains was approved in 2006, but only part of the global female population is vaccinated [242]. HPV belongs to the non-enveloped DNA viruses, and the viral proteins E6 and E7 have oncogenic activity or contribute to malignant progression [243]. In high-risk HPV strains,

E7 has transforming activities, and the tumour suppressor p53 can be inactivated by E6, preventing p53 from inducing apoptosis, which makes both proteins interesting as drug targets [244, 245]. There have been reports of using intracellularly expressed antibodies to influence oncogenic effects of the viral proteins E6 as well as E7 [246–250]. Interestingly, the epitope recognized by an antibody directed against E6 was found to be in a region that could hinder recognition of the E6 nuclear export signal. As hypothesized by the authors, this could be a potential mechanism by which export of E6-bound p53 to the cytosol might be blocked, and, as a consequence, proteasomal degradation of the tumour suppressor p53 might be prevented [249]. The strategy to influence the subcellular localization of a protein for modulating its function, or as hypothesized here to influence turnover as a consequence of trapping in the nucleus, allowed studying cells in an entirely new way. The prevention of p53 degradation was also achieved in HPV-infected cells by an intracellularly expressed nanobody. In contrast to the aforementioned example, the intracellular nanobody did not bind E6 but was specific for the DNA binding domain of p53. Although the tumour suppressor p53 was stabilized against degradation by the nanobody, the function of p53 was inhibited upon expression of the nanobody, resulting in increased cell proliferation [251]. This impressively demonstrates how intrabodies can be used to study different functional details of the exact same process, in this case by targeting a different binding site. The therapeutic potential of intrabodies in HPV treatment was further explored by Amici and colleagues [250]. An E6-specific intrabody was isolated using IACT, a method for selecting antibodies that fold correctly in the cytoplasm. The E6-specific intrabody was expressed and directed to the nucleus in cells. As a result, p53 accumulated in the nucleus of SiHa cells and was able to partially inhibit its degradation. Via a retroviral vector, the E6 intrabody was expressed in preclinical mouse models of HPV and resulted in a marked delay of tumour onset. While all mice had developed tumours by 20 days after injecting HPV16-positive tumour cells, 60% and 40% of mice injected with TC-1 and C3 intrabody-expressing tumour cells, respectively, remained tumour free for more than 4 months [250].

5.1.5 Hepatitis B Virus

Hepatitis B virus (HBV) is an enveloped DNA virus with a virion diameter of approximately 45 nm and a 36 nm diameter core [252]. HBV can be prevented by prophylactic vaccines, but vaccination is not sufficiently effective to induce seroprotection for every person. Approximately 5% of the population does not respond sufficiently to vaccination [253] and HIV patients in particular have lower response rates [254, 255]. The regional prevalence of HBV infection varies

[234, 256]; with a prevalence of 10–25% in some developing countries, HBV is still a concern [257]. HBV infection can result in various liver disorders or hepatocellular carcinoma, which still causes 500,000–700,000 deaths per year [253, 258, 259]. Expression of a nanobody as an intrabody in the cytosol or nucleus was used to attack the HBV core protein (HBcAg) [260]. The secreted viral proteins hepatitis B surface antigen (HBsAg) and hepatitis B e-antigen (HBeAg) have been retained in the ER by ER intrabodies. HBeAg secretion was downregulated by the ER intrabody, but HBcAg was not affected, although its sequence is similar, and cross-reactivity of the anti-HBeAg antibody to HBcAg was shown [261]. This demonstrates how proteins with high sequence similarity but residing in different compartments can be targeted separately from each other by employing the compartment-specific action of intrabodies. An ER intrabody specific for HBsAg was shown to inhibit secretion of HBV virions in vivo in a mouse model, demonstrating the therapeutic potential of this approach [259].

5.1.6 Hepatitis C Virus

Hepatitis C virus (HCV) causes approximately 400,000 deaths per year as a consequence of chronic HCV-induced liver disease and liver carcinoma, in spite of advances in HCV treatment [262, 263]. HCV is an enveloped (+)-strand RNA virus that produces viral components as a polyprotein that needs to be cleaved into its individual functional protein units [264]. HCV core protein, which forms the viral capsid [264], was the target of intracellularly expressed antibodies [265–267]. Despite the availability of direct-acting antivirals and small-molecule inhibitors for HCV treatment, the high mutation rate of HCV can rapidly lead to the emergence of drug resistance in patients during treatment [268, 269]. The inhibitory effect observed upon expression of an NS3-specific intrabody was maintained even in the presence of point mutations that confer resistance to small-molecule drugs. As hypothesized by the authors, resistance to antibody-based drugs might occur more slowly than resistance to small-molecule-based drugs because antibodies contact their targets over a comparably large surface area via multiple residues [270].

5.2 Targeting Host Processes in Viral Infections

Viral infections are counteracted by the host protein interferon (IFN)-α, but due to the high homology of the 14 different isoforms of IFN-α in mice, generating knockout mice on a genetic level would require immense effort. A single intrabody that recognizes all isoforms could provide a solution. Following this hypothesis, an ER intrabody specific for IFN-α isoforms was expressed in different cell lines in vitro and allowed blocking of IFN-α secretion as well as increased

virus proliferation as a consequence of ER intrabody-mediated IFN-α knockdown [58]. Knocking out many genes with similar functions to generate a mouse model may no longer be required if all isoforms of a protein can be targeted with a single intrabody, which means only a single gene has to be integrated into the genome to generate a transgenic intrabody mouse. Because intrabody expression is dominant, a marked phenotype may already be observed in an earlier stage during the generation of a mouse model: heterozygous intrabody mice might already display sufficient knockdown of the target protein, in contrast to a heterozygous genetic knockout of the target. Intrabodies could in this way markedly accelerate the generation of mouse models.

6 Applications in Oncology

A phase I clinical trial was performed to assess the feasibility of adenovirus-mediated intrabody gene therapy [271], and various strategies have been suggested for the intracellular use of antibodies to target processes related to cancer (Table 5).

6.1 Applications in Oncology: Targeting at the Cellular Level

At the cellular level, intrabodies have been used to interfere with protein–protein interactions in oncogenic pathways [272] and to influence processes involved in proliferation, the cell cycle and apoptosis. DNA repair and genomic stability were studied by a nanobody fused to a fluorescent protein that allowed tracking of poly (ADP-ribose) polymerase 1 (PARP1), a target for cancer therapy to inhibit DNA repair for sensitizing tumour cells to radio- or chemotherapy [273]. Several proof-of-concept studies have demonstrated downregulation of human epidermal growth factor receptor-2 (HER2) or inhibition of cell proliferation by intrabodies targeting HER2 [272, 274]. A nanobody directed against the tumour suppressor p53, which is involved in processes such as the cell cycle and apoptosis [275], was used to delocalize p53 by capturing it at mitochondria to generate a functional knockdown [64]. By the expression of a library of intrabodies in tumour cells, potential mechanisms by which cells are rescued from apoptosis as a contribution to cancer progression were identified [276]. The capability of intrabodies to specifically target post-translational modifications was demonstrated by an intrabody against a particular phosphorylated form of signal transducer and activator of transcription 3 (STAT3), which plays a role in various processes including proliferation and apoptosis [277]. STAT3 can be phosphorylated at different sites or remain unphosphorylated. By using an intrabody, it was possible to block the function of the tyrosine-phosphorylated form of

STAT3 (pYSTAT3) but not the serine-phosphorylated form (pSSTAT3) or the unphosphorylated form (USTAT3), which is impossible for conventional small molecule-based inhibition. This was demonstrated both in vitro and in mice and illustrates the potential of intrabodies to study mechanisms based on individual functional units of proteins [278].

6.2 Applications in Oncology: Targeting at the Tissue Level

At a level superordinate to cells, processes such as adhesion, metastasis and angiogenesis have been targeted by intrabodies. As adhesion molecules, integrins have implications in metastasis [279], and, therefore, a better understanding of integrin function might be beneficial for cancer research. Integrins can form different heterodimers depending on which integrin subunits combine. The retention of a particular integrin subunit in the ER by an intrabody allowed study of how integrin heterodimers form and revealed a hierarchy in complex formation when different subunits combine [280]. Tumour invasion and metastasis were further targeted using an intrabody to block the activation of matrix metalloproteinases in an in vitro model [281, 282]. Plasma membrane extensions that also have proteolytic activity, called invadopodia, allow cancer cells to invade their surroundings and form metastases. The protein cortactin is involved in invadopodia formation, and a better understanding of this potential drug target was achieved by blocking the function of individual domains of the protein by intracellular nanobodies, which revealed different outcomes when different epitopes of cortactin were targeted [283, 284]. The GTPase RHOB is also involved in invasion. A conformation-specific intrabody that was targeted for degradation allowed selective degradation of only the guanosine triphosphate (GTP)-bound form of the protein. Targeted degradation with this conformation-specific intrabody revealed the processes of invasion and genomic instability to be associated with only one but not the other conformation of RHOB [132]. These examples impressively illustrate how intrabodies allow research on the functional consequences of complex formation in the presence of different subunits that complex in different combinations, blocking individual protein domains and individual conformations of a protein. Angiogenesis, the process of forming new blood vessels, has been successfully blocked by ER intrabodies specific for vascular endothelial growth factor receptor 2 (VEGFR2) in in vitro models [285, 286], and a bispecific intrabody allowed inhibiting angiogenesis and tumour growth by targeting VEGFR2 and the angiopoietin receptor Tie2 [287]. An adenovirus that delivered Tie2 intrabody to mice allowed a marked reduction in vessel density and significant reductions in two types of tumours, which were xenografts derived from human Kaposi's sarcoma or human colon carcinoma [288]. The

therapeutic potential of intrabodies with respect to angiogenesis may in the future be extended by broadening the range of angiogenesis-related targets attacked by intrabodies. In addition to the strategy of starving tumours by blocking angiogenesis, the normalization of tumour vascularization has emerged as another therapeutic strategy because vascularization is expected not only to supply the tumour with nutrients but also to allow the supply of chemotherapeutics, making chemotherapy more effective [289].

6.3 Applications in Oncology: Targeting Neo-Antigens

Neo-antigens or oncoproteins, aberrant proteins that exist only in tumour cells, are particularly interesting targets because they allow the often difficult discrimination of tumour and healthy cells during therapy. The oncoprotein Bcr-Abl originates from the fusion of two genes due to the formation of an aberrant chromosome, which results in a constitutively active kinase associated with chronic myelogenous leukaemia [290]. Because intrabodies can be used to de-localize proteins, an intrabody was employed to re-route Bcr-Abl to the nucleus, where it causes apoptosis. To obtain Bcr-Abl-specific intrabodies that are functional in the cytosolic milieu, IACT was employed for intrabody selection [291]. The protein Ras, which is involved in signal transduction and is frequently mutated in cancer, can become permanently activated due to mutations and influence cell growth and survival [292]. Intrabodies targeting Ras were already developed in the early 1990s by Cattaneo and colleagues [293, 294]. In a new concept, antibody–antigen interaction-dependent apoptosis (AIDA), an intrabody specific to mutant Ras, was split into two parts variable heavy chain (V_H) and variable light chain (V_L). Each part was fused to inactive procaspase-3 and auto-activation of caspase-3. The formation of a tri-molecular complex of mutant Ras and the two procaspase-3-fused intrabody halves allowed the induction of apoptosis upon the binding of mutant Ras. This method could be further extended to any neo-antigen for which a specific intrabody is available [295]. A different approach was taken by using intrabodies for target validation to block protein–protein interactions in disease models and subsequently find small molecules that overlap with the antibody binding site. An anti-mutant Ras intrabody allowed the identification of compounds from a small-molecule library that are able to mimic the effect of the intrabody on protein–protein interactions [296].

6.4 Applications in Oncology: Targeting Tumours Using Cellular Immunotherapy

There are recently approved cellular therapies for haematological diseases based on immune cells that are directed towards killing tumour cells [297]. The direction of immune cells as weapons against tumour cells is achieved by means of chimeric antigen receptors (CARs) on T cells or natural killer (NK) cells. CARs are expressed after ex vivo genetic engineering of cells, which are subsequently reinfused into patients [298, 299]. Ex vivo genetic engineering of lymphocytes for cellular therapy has recently also been pursued for intrabodies. As a target for CAR T cell therapy, CD7 was chosen due to its consistent expression in T cell acute lymphoblastic leukaemia. However, CAR T cells expressed CD7 as well, which resulted in killing of not only tumour cells but also of CAR T cells. To prevent this, an ER intrabody was employed to deplete expression of CD7 in CAR T cells. The expression of the ER intrabody prevented self-attack by CAR T cells and allowed them to kill only leukaemic cells [44].

7 Summary and Comparison of all Strategies

The expression of intrabodies in the cytosol or nucleus usually requires an involved selection procedure, but technologies have been developed to address this requirement and have allowed the generation of cytosolic intrabodies for many research areas [89, 95]. The generation of ER intrabodies is relatively straightforward, allowing more freedom in the choice of the epitope, and the folding properties of normal antibodies are sufficient. ER intrabodies have so far been found to be well-tolerated by cells and in mice. Even substantial overexpression of an ER intrabody specific for the neuronal receptor p75NTR was not found to induce ER stress, as indicated by the absence of induction of the unfolded protein response (UPR) [300], and no marked ER stress was found by other researchers examining the retention of Z alpha antitrypsin by an ER intrabody [301]. Knockdown of VCAM1 by an ER intrabody was also detected in living mice [302], and expression of the ER intrabody by transgenic mice was well-tolerated [57]. The delivery of antibodies to the cytosol as proteins is no longer a challenge in vitro but reliably applicable with standard laboratory equipment (see also Table 3). Whether there is therapeutic potential for protein delivery still needs to be further explored. Delivering proteins to endosomes or lysosomes is less challenging but may raise the question of what therapeutic effect could be obtained by targeting these compartments, especially because the delivered protein is degraded in the lysosome. A particularly interesting future application of delivering antibodies to lysosomes could be in the context of intracellular pathogens that inhibit lysosomal acidification.

In addition to the possibility of inhibiting POIs by neutralization or re-localizing POIs, all methods based on intrabody expression or antibody delivery to the cytosol allow

the targeting of POIs for degradation (Table 6). Intrabodies have already been delivered by gene therapy in preclinical animal models. The delivery of proteins to lysosomes is readily achievable even in vivo and has been used for drugs that have already been approved for therapy, including an immunotoxin [55] and ADCs [303–306].

8 The Future: Key Preclinical and Clinical Challenges

Using intrabodies for therapy is interdisciplinary, requiring expertise in intrabody-related technologies based on molecular and cellular sciences, in specific disease areas and in clinical application. This may in some cases pose a higher threshold to using this approach. However, for certain applications, there might be no suitable or feasible alternatives [8, 307]. In previous decades, the laborious and still error-prone technologies for antibody generation have complicated the use of intrabodies. This former hurdle may be responsible for the comparably late increase in research articles in this field. Antibody generation technologies and selection technologies for cytosolic intrabodies are no longer limitations. A large number of well-validated and sequence-defined antibodies are ready for use as intrabodies and delivery as proteins into cells.

There are also a variety of successful strategies to degrade a POI. A limited number of small-molecule reagents are available to bind POIs, and targeting protein–protein interactions has been reported to be challenging for small molecules, which are small and have little contact area with the protein [12–15]. Targeted degradation by protein binders would therefore have enormous potential, but therapeutic application will critically depend on the feasibility of in vivo protein delivery. Local in vivo electroporation delivery of antibodies has been performed in rats [37], and in vivo electroporation has been applied in further model organisms, e.g. locally to muscle tissue [308]. In addition to in vivo delivery to tissues, ex vivo delivery for cell therapy could be explored as an application for protein delivery in the future. For research purposes, delivering antibodies to the cytosol might become a standard method in many research areas, depending on the availability of reagents that are affordable and suitable for this application.

The application of intrabodies in clinical use depends on in vivo or ex vivo gene therapy. Particularly in the area of neurodegenerative diseases, there are comparably many preclinical in vivo studies, probably due to the urgent necessity of targeting pathologic proteins. Pathologic mechanisms associated with protein conformation are more amenable to being targeted by antibodies than by classic small-molecule therapy, which works better for inhibiting enzymes. Gene therapy to correct the gene for mutant huntingtin (*mHtt*) cannot remedy pathogenic mHtt protein because it may have prion-like properties and convert other proteins to pathogenic forms. Intrabodies could solve this problem. A range

Table 6 Comparison of strategies to target the inside of cells with antibodies

Properties	Intrabody expression		Antibody delivery (as a protein)	
	Cytosol/nucleus	ER/mitochondria	Cytosol/nucleus	Other compartments
Correct antibody folding possible?	?	+	+	+
Epitope choice: can non-neutralizing antibodies be used?	− (+ if combined with targeted degradation)	+	− (+ if combined with targeted degradation)	− (+ if combined with targeted degradation)
Antibody-mediated functional knockdown	+	+	+	?
Targeted degradation?	Tags targeting for proteasomal degradation	ER: targeting for ERAD	TRIM21 for antibodies with Fc part, tags targeting for proteasomal degradation	Lysosomal targeting (e.g. LYTACs)
Examples of special features targeted	PTMs, individual domains, conformations	Complex formation, knockdown in selected cellular compartment	Long half-life protein degraded, tracking of unmodified endogenous POIs	
Deliverable in therapy?	Adoptive cell therapy, gene therapy	Adoptive cell therapy, gene therapy	Adoptive cell therapy? Local tissue electroporation?	+ (ADCs, approved for therapy)

ADCs antibody–drug conjugates, *ER* endoplasmic reticulum, *ERAD* ER-associated degradation, *LYTACs* lysosome-targeting chimaeras, *POIs* proteins of interest, *PTM* post-translational modification, *TRIM21* tripartite motif containing-21,? indicates to be determined, − indicates no, + indicates yes

of preclinical animal models have demonstrated the feasibility of intrabody gene therapy, and intrabody expression has been well-tolerated in animals [57]. This might not be surprising considering that high expression of antibodies during an immune reaction should be well-tolerated. Challenges might still arise from disease-specific pathologic mechanisms, which require a more detailed understanding of the pathologic process as well as the exact functional effects of individual intrabodies on their targets. While many intrabodies block the aggregation of pathologic proteins, the aggregation rate has also been found to be increased by an intrabody [309]. Additionally, two different intrabodies have been reported by the same authors, of which one accelerated the mutant phenotype and one rescued it [310]. In cancer research, several different Bcr-Abl-specific intrabodies with a signal sequence for nuclear translocation differed in their effect on the re-localization of Bcr-Abl to the nucleus [291]. A related observation was made by Martinelli and colleagues [311]. A better mechanistic understanding of how function is affected by binding of the antibody to its target in the cell might therefore be required to select for antibodies with the intended effects. Intrabodies targeting individual post-translational modification (PTM)s could in the future contribute further to a better understanding of processes in health and disease [312, 313]. Cellular therapy employing an ER intrabody has recently been proposed as a therapeutic approach [44]. Because it provides a solution to the challenges of intrabody delivery that has been demonstrated to be feasible in approved CAR T cell therapies [297], this approach might be a particularly promising avenue for therapeutic application of intrabodies in the future.

Compliance with Ethical Standards

Conflict of interest CZ is named as an inventor on patents and patent applications in the field of cancer immunotherapy owned by his previous institution (Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt am Main, Germany). CZ declares that this review article was written in the absence of any other commercial or financial relationships that could be construed as a potential conflict of interest. RMÖ, JR, RM and ALJM report no conflicts of interest.

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