Refined immunoRNases for the efficient targeting and selective killing of tumour cells: A novel strategy

Mohammadreza Nassiri, Reihane Behnam-Rasouli, Masoume Vakili-Azghandi, Vinod Gopalan, Peyman Dolati, Rouhollah Nourmohammadi

PII: S0024-3205(21)01209-1

DOI: https://doi.org/10.1016/j.lfs.2021.120222

Reference: LFS 120222

To appear in: *Life Sciences*

Received date: 19 September 2021

Revised date: 13 November 2021

Accepted date: 5 December 2021

Please cite this article as: M. Nassiri, R. Behnam-Rasouli, M. Vakili-Azghandi, et al., Refined immunoRNases for the efficient targeting and selective killing of tumour cells: A novel strategy, *Life Sciences* (2021), https://doi.org/10.1016/j.lfs.2021.120222

Refined immunoRNases for the efficient targeting and selective killing of tumour cells: A novel strategy

Mohammadreza Nassiri[1,2*], Reihane Behnam-Rasouli[1], Masoume Vakili-Azghandi[3], Vinod Gopalan[4], Peyman Dolati[5], Rouhollah Nourmohammadi[6]

[1] *Recombinant Proteins Research group, The Research Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran*

[2] *School of Life and Environmental Sciences, The University of Sydney, Sydney 2006, NSW, Australia*

[3] *School of Pharmacy, The University of Sydney, Sydney 2006, NSW, Australia*

[4] *Cancer Molecular Pathology, School of Medicine, Griffith University, Gold Coast, Queensland 4222, Australia*

[5] *Institute for Marine and Antarctic Studies (IMAS), University of Tasmania, Hobart, Tasmania, Australia*

[6] *Department of Animal Science, College of Agriculture, Lorestan University, Khorramabad, Lorestan 68137–17133, Iran*

[*]**Correspondending author at:** School of Life and Environmental Sciences, The University of Sydney, Sydney 2006, NSW, Australia

E-mail address: nassiryr@um.ac.ir (M.R. Nassiri).

Abstract

In order to overcome limitations of conventional cancer therapy methods, immunotoxins with the capability of target-specific action have been designed and evaluated pre-clinically, and some of them are in clinical studies. Targeting cancer cells via antibodies specific for tumour-associated surface proteins is a new biomedical approach that could provide the selectivity that is lacking in conventional cancer therapy methods such as radiotherapy and chemotherapy. A successful example of an approved immunotoxin is represented by immunoRNases. ImmunoRNases are fusion proteins in which the toxin has been replaced by a ribonuclease. Conjugation of RNase molecule to monoclonal antibody or antibody fragment was shown to enhance specific cell-killing by several orders of magnitude, both in vitro and in animal models. There are several RNases obtained from different mammalian cells that are expected to be less immunogenic and systemically toxic. In fact, RNases are pro-toxins which become toxic only upon their internalisation in target cells mediated by the antibody moiety. The structure and large size of the antibody molecules assembled with the immunoRNases have always been a challenge in the application of immunoRNases as an antitoxin. To overcome this obstacle, we have offered a new strategy for the application of immunoRNases as a promising approach for upgrading immunoRNAses with maximum affinity and high stability in the cell which can ultimately act as an effective large-scale cancer treatment. In this review, we introduce the optimized antibody-like molecules with small size, approximately 10 kD, which are presumed to significantly enhance RNase activity and be a suitable agent with the potential for anti-cancer functionality. In addition, we also discuss new molecular entities such as monobody, anticalin, nonobody and affilin as refined versions in the development of immunoRNases. These small molecules express their functionality with the suitable small size as well as with low immunogenicity in the cell, as a part of immunoRNases.

Keywords: ImmunoRNase, Protein Scaffold, Antibody, Cancer

1. Introduction

Due to disruptive side effects of current therapeutic methods, selective cytotoxicity has been a major objective for anti-cancer therapy, and antibody technology has led to significant target-cell selectivity [1]. ImmunoRNases, as novel selective drugs in cancer therapy, can target tumour cells, as these agents have exhibited efficient tendency to create a tumour-specific chemical bond to the surface proteins of cancer cells [2, 3]. ImmunoRNase molecules are a group of chimeric proteins that consist of a monoclonal antibody or antibody fragment

conjugated to a cytotoxic agent like RNases [4]. Structural studies indicate that the immunoRNases have three principal domains including: (a) a cell-binding domain that attaches the antibody to the surface of the tumour cell; (b) a translocation domain that transfers the toxin molecule to the cytoplasm of the targeted cell; and (c) a catalytic domain that modulates various cellular processes like cell death [5, 6]. A combination of the three domains of the immunoRNases enables them to selectively deliver ribonucleases, thus replacing the use of chemotherapy. Most of the chemicals currently used for anti-tumuor purposes trigger apoptosis by inducing DNA damage and disrupting the cell cycle. However, immunoRNases induce cell death with no mutagenic impacts on the cell cycle [7].

More recently, small protein scaffolds have been proposed as ideal molecules for the treatment of cancer and infectious diseases, mainly because they do not possess any disulfide bonds and rigid structure that hinder the transfer and internalization of immunoRNAses to specific cancer cell receptors [8]. The first reason for not using full antibodies is their large size, ranging from 50 to 150 kD, which makes internalization to cancer cells very difficult [9]. Another major obstacle is the side effects of full-length antibodies [10]; therefore, research has been directed at finding smaller molecules that can substitute for the whole immunoRNase, which can improve the efficacy of the existing therapies. We can produce an optimized antibody-like molecule of approximately 10 kD, or even smaller in size, to improve the permeability efficiency [11]. The effective structure is smaller in size and has lower immunogenicity than antibodies. Due to the fact that small protein scaffolds do not possess any disulfide bonds and their structure is flexible enough for integrating into the targeted cells, they are considered to be a suitable replacement for a complete immunoRNase.

To our knowledge there is no other study on the application of immunoRNase with small protein scaffolds. In this review we will focus on human protein scaffolds.

2. Toxins

Protein toxins are characterized as highly active enzymes that have shown the potential to destroy tumours. The toxins can be obtained from herbal, bacterial or animal sources, but they all have a similar mechanism of action in suppressing protein translation, which is by inducing the apoptosis signalling pathway [12]. A tumour cell is targeted explicitly by the immunotoxin in which the toxin and antibody are linked to a specific tumour-associated antigen. Functionally, after entering the cells, the conjugated immunotoxin will selectively kill the tumour cells [13]. Toxicity and immunogenicity are two contributing factors that have complicated the application of immunotoxins. Theoretically, the toxin segment of the

immunotoxin can be replaced by a non-toxic RNase.The major drawback for the further clinical development of therapeutic fusion animal RNases is immunogenicity, in addition to the side effects for patients [14]. Theoretically, the toxin segment of the immunotoxins can be replaced by a non-toxic RNase.

The original functional immunotoxins were produced from intact antibodies attached to plant toxins like ricin, saporin, or gelonin [15]. In the second generation, bacterial toxins like diphtheria toxin, pseudomonas exotoxin, and anthrax toxin were fused with the antibody moiety. The bacterial toxins contribute to inhibition of protein translation by inducing ADP ribosylation of EF2 factor, resulting in cellular apoptosis [12]. Despite the high potency of bacterial and plant toxins, they have some problems that restrict their therapeutic application. Exogenous toxins elicit an immune response that resulted in a decreased half-life. [12, 16]. To overcome these difficulties, the endogenous RNase enzymes from human and animal sources were conjugated with targeting fragments to produce a new generation of immunoRNase with low immunogenicity [17].

3. Immunotoxin generations

The first-generation immunotoxins were constructed by the combination of pseudomonas exotoxin chain and a full-length monoclonal antibody. The antibody and the toxin proteins were fused by disulfide or thioether bonds. If the whole toxin chain was used, this immunotoxin had low specificity, as it attached to normal cells as well as tumour cells [12]. However, the severe hepatotoxicity and immunogenicity of the toxin part of these chimeric proteins restricted their application in cancer therapy [18]. To overcome the non-specificity of these immunotoxins, the binding domain of toxin molecules was removed. This significantly increased the specificity of the immunotoxins, since the truncated toxin could no longer bind to normal cells. However, the size of these proteins was still large enough to limit their functionality for tumour penetration, especially in solid tumour cells [19]. To reduce the specificity limitation of immunotoxins, the next generation of immunotoxins was developed by recombinant DNA technology. In the 1990s, molecular cloning techniques were used to produce the third generation, known as recombinant immunotoxins To date, more than 1,000 immunotoxins from the third generation have been produced [20].

Recombinant immunotoxin constructs were made from human antibody fragments conjugated with the toxic catalytic domain [12]. Conjugated proteins target tumour cells by selectively binding to the surface antigens that are only expressed on cancer cells (**Fig. 1**) [21-23]. Using herbal or bacterial toxins in these conjugate constructs can induce severe

immunologic responses that limit their action against tumour cell growth. To overcome these problems, non-toxic animal proteins are proposed for conjugation with the antibodies for the formation of the recombinant immunotoxins [22].

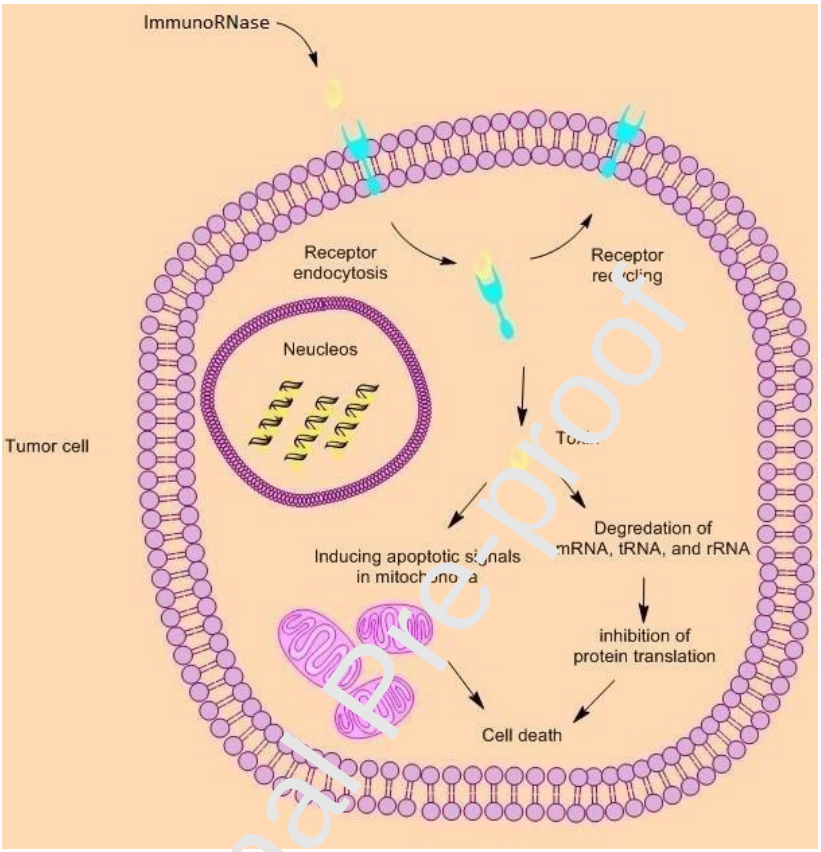


Fig. 1. Mechanism of action of immunotoxins to induce cell death in cancer cells. Two mechanisms of action of immunoRNase for killing cancer cells are (a) inhibition of protein translation, and (b) inducing apoptotic signals in mitochondria.

3.1. ImmunoRNases

The ribonuclease (RNase) enzyme family is one of the most frequently used animal toxins. It is composed of various enzymes that are capable of cleaving the phosphodiester bonds in mRNA, tRNA and rRNA molecules. The RNase enzymes also contribute to several physiological functions, including anti-tumour, antibacterial, and antiviral activities [4]. The RNase enzymes are able to change gene expression machanisms and reprogram the transcriptome of targeted tumour cells to terminate their translational regulation and cause cell apoptosis. The cytotoxic activity of RNase enzymes was found to be initiated only after their

internalization to the targeted cells by inducing cell cycle arrest and suppressing cellular proliferation [23, 24].

ImmunoRNases are high-potency chimeric proteins that specifically target the surface antigens on cancer cells. However, there are several limitations in using immunoRNases in cancer treatment. For instance, due to the low expression of surface antigens on tumour cells, non-tumour cells may also be targeted by immunoRNases. Recent efforts to identify cancer cell-specific antigens seek the potential for designing novel immunoRNases capable of targeting tumour cells with high specificity and lower toxicity [25, 26].

Tumour cells are specifically targeted by the immunotoxin. After entering the cells, the conjugated part of the immunotoxin will selectively kill the tumour cells. The toxicity and immunogenicity are two main contributing factors that always have been considered as the challenges of the clinical application of immunotoxins [27]. A significant problem for the further clinical development of therapeutic fusion of the animal RNases is immunogenicity and side effect for patients. The toxin segment of the immunotoxins can be replaced by a non-toxic RNase. The target cells can be selected by the immune moiety of an immunoRNase (**IR**) when passing through the bloodstream, with no damaging effect to cells devoid of the targeted tumour-associated antigen [23]. As soon as the immunotoxins are internalized, the RNA degrading activity will be used, leading to cell death. Non-toxic and non-immunogenetic effects of IR are strengthened when containing both a human RNAase and human antibody fragment. The cytosolic RNase inhibitor (**RI**) can be neutralized by flooding the cytosol with high levels of IR, which result in neutralizing the RI, or by using RNases resistant to the inhibitor [14]. Another approach to developing more effective RNases is to use genetic and protein engineering. We postulated that replacing amino acids in the HP-RNase 1 surface loop might lower the interaction with RI and improve cytotoxicity. The surface loop is important in conformational consistency and ribonucleolytic activity and also reduces the inhibitory effect of RIs in mammalian cytosol [28].

3.1.1. Human RNases with therapeutic potential

Several RNase enzymes like human pancreatic RNase, eosinophil derived neurotoxin, and eosinophil cationic protein have been utilized for fusion with various targeting fragments in order to produce new immunoRNases with low immunogenicity and toxicity to normal cells. These RNase enzymes are able to inhibit tumourigenesis. Their toxicity and specificity for cancer cells were significantly enhanced after conjugation with the antibodies as targeting

fragments [4, 5]. The list of reported RNase enzymes that were used in clinical studies is shown in **Table 1**.

Table 1. List of the reported RNase enzymes in clinical studies with RNase-based immunotoxins that demonstrate efficacy in different cancer types.

ImmunoRNase	Source	Cancer tested	Targeting moiety	Reference
Human pancreatic RNase1 –EGF	pancreas	squamous cell carcinoma	Epidermal growth factor	[29]
Eosinophil cationic protein – scFv	eosinophils	oral and bladder tumours	Anti-TFRC scFv	[30]
Eosinophil-derived neurotoxin –scFv	Eosinophil, spleen and liver	breast, lung, leukemia, and ovary cancers	Anti-TFRC scFv	[31, 32]
Angiogenin –EGF	Tumour cells	colon, gastric, hepatocellular, pancreatic, and endometrial cancers	Epidermal growth factor	[33, 34]
Onconase -V3	oocytes	mesothelioma, breast, and renal cell cancers	Anti-CXCR4 scFv	[35]
BS-RNase–scFv	bovine seminal fluid	lung, thyroid cancers	Anti-Lewis Y scFv	[36]

3.1.1.1. Human pancreatic RNase1

The human pancreatic RNase1 enzyme is expressed in pancreas and other organs. The mechanism of physiologic action of human pancreatic RNase1 is not known [29]. It has been suggested that this enzyme is devoid of any role in tumour penetration or in any other biological processes that may cause some unexpected side effects. The cytotoxicity of pancreatic RNase1 enzyme was increased when conjugated with antibody fragments. There are several immunoRNases with human pancreatic RNase1 developed against various malignancies like squamous cell carcinomas [24, 30]. Over the last decade we did some RNase family engineering with the substitution of suitable amino acids. In 2014, our group located some amino acids with the potential to affect interaction with the inhibitor in HP-

RNase 1 structure and exposed them to replacement mutagenesis [37]. We hypothesized that amino acid replacement in the HP-RNase 1 surface loop could significantly improve its conformational stability and ribonucleolytic activity and decrease inhibitory activities of RIs in cytosol of mammalian cells. By manipulating the interaction between RI and RNase 1, it might be possible to produce other recombinant variants of HP-RNase 1 via replacing different amino acid in a different position to degrade interaction with RI and improve cytotoxic activity. We hypothesised that the replacement of amino acids of HP-RNase 1 in the surface loop could improve their conformational stability and ribonucleolytic activity and also decrease the inhibitory activities of RIs in mammalian cytosol [37].

3.1.1.2. Eosinophil-derived neurotoxin (RNase2)

The eosinophil-derived neurotoxin (**EDN**), also known as RNase2, is expressed in eosinophils and other organs, including the spleen and liver [31]. EDN exhibits some toxic activities such as antihelminthic, antiviral and neurotoxic activities. The mechanisms behind the identified physiological functions and enzymatic activities of EDN are poorly understood. The catalytic activity of RNase2 is significantly higher than RNase3 [38, 39]. The RNase2 enzyme was conjugated with antibody fragments in the immunoRNA constructs and was tested against multiple human malignancies, including breast, lung, leukemia and ovary [40].

3.1.1.3. Eosinophil cationic protein (RNase3)

The eosinophil cationic protein is also known as ribonuclease 3 (**RNase3**). It also has antihelminthic, antiviral and neurotoxic activities similar to RNase2 but with a lower catalytic efficiency. Several studies have indicated that RNase3 has high stability in comparison with the other RNase enzymes. Moreover, it has an anti-proliferative role for controlling the growth of cancer cells. Having these special characteristics, RNase3 can be used as a catalytic partner in novel immunoRNases for different human cancer therapeutic purposes [32, 41].

3.1.1.4. Angiogenin

Angiogenin, or RNase 5, is implicated in the processes of rRNAs in angiogenesis of normal and tumour cell growth. Because of high metabolic activity in tumourogenisis, the expression of angiogenin is normally upregulated in tumour cells [33]. This leads to neovascularization and tumour growth. Recent studies have revealed that the over-expression

of angiogenin is correlated with tumour progression and aggressiveness, suggesting that angiogenin can be used as a prognostic biomarker in various malignancies including colon, gastric, hepatocellular, pancreatic, and endometrial cancers [42, 43]. Similar to other RNases, angiogenin has no cytotoxic effects by itself, but when it is conjugated with an antibody and translocated to the target cells, it can induce cellular apoptosis through hydrolyzing tRNAs and suppressing protein translation [5].

3.1.2. Onconase

In addition to RNase enzymes obtained from human sources, there are some RNase enzymes that are from other animal sources [5]. The onconase (ONC) or ranpirnase enzyme is a pancreatic ribonuclease that was first derived from the oocytes of the northern leopard frog [44]. ONC enzymes exhibit cytotoxic activities to human cancer cells by degrading t-RNA and double-stranded RNA molecules that result in death of tumour cells and/or apoptosis [35]. The ONC enzyme can be transferred into tumour cells through binding to cell surface receptors. Then ONC inhibits the progression and development of tumour cells by disturbing the transcription mechanism. The anti-cancer activity of ONC enzymes was investigated in clinical trials for several human malignancies like mesothelioma, breast, and renal cell cancers [45, 46]. ONC had non-specific cytotoxic effects like spermatogenic, embryotoxic and nephrotoxicity in cancer patients. Recent studies indicate that, in combination with standard chemotherapeutic drugs, ONC can exhibit synergistic effects against human cancers. To further overcome the side effects of ONC enzymes, the conjugation of ranpirnase with an antibody specific for EGFR-positive tumours may result in better clinical outcomes with less side effects on cancer patients [45]. We published research on ONC engineering [27], and the primary goal of this study was to modify ranpirnase and to estimate engineered ranpirnase characteristics such as cellular uptake, cytotoxicity, the ability to avoid RI, and functionality of the protein.

3.1.3. BS-RNase

The bovine seminal RNase enzyme (**BS-RNase**) is the only enzyme with a quaternary structure that contains two of the same subunits held together with two disulfide bonds. The anti-tumour function of the BS-RNase enzyme is mediated by its enzymatic degradation of rRNA molecules, leading to suppressing protein synthesis in cancer cells [47, 48]. The cytoxicity of BS-RNase to the tumour cells is mainly due to the ability of this enzyme to neutralize the activity of RI. BS-RNase shows cytotoxic activity against malignant cells *in*

vitro and *in vivo* [48]. In addition to its anti-tumour function, the BS-RNase enzyme has anti-spermatogenic and immunosuppressive activities. RNase enzymes may suppress tumour cell growth and metastasis by inducing cellular apoptosis [49, 50]. We engineered a bovine pancreatic ribonuclease (**RNase A**) for combination with cetuximab. To decrease RNaseA binding to RI, six amino acids including alanine, aspartic acid, aspartic acid, alanine, arginine, and alanine were substituted for six amino acid residues at lysine 7, arginine 39, asparagine 67, asparagine 71, glycine 88, and glutamic acid. Then the major features of engineered-RNase A were investigated. These outcomes demonstrated that engineered-RNase A can both preserve its structures and features and decrease binding to cytosolic RI [51].

3.1.4. Application of immunoRNases (IR) in cancer therapy

In recent years, immuno-oncological approaches have significantly changed cancer therapy. Several biopharmaceuticals are currently being evaluated for the treatment of a broad range of cancer tumours. Erb-hcAb-RNase, a novel human IR, was constructed by fusing compact anti-ErbB2 receptor antibody and HP-RNase with anti-cancer activity against the Her2 receptor [52]. This study demonstrated that Erb-hcAb-RNase preserves the enzymatic activity of HP-RNase and explicitly binds to ErbB2-positive cells with high affinity. This type of IR acts effectively and selectively for ErbB2-positive tumour cells, in *in vitro* and *in vivo* studies, and it has more anti-tumour activity than the parental Erb-hcAb. ERB–HP-DDADD-RNase, as the second-generation anti-ErbB2 IR, is constructed by fusing erbicin (a human ErbB2-directed scFv) with an inhibitor-resistant variant of RNase 1 (HPDDADD- RNase) [53]. This IR (ERB–HPDNase) has the enzymatic activity of RNase 1 and the specific binding of the parental scFv to ErbB2-positive cells.

Other results indicated that the conjugation of RNase (including wild, engineered and HP-RNase) with the antibody to the IR can improve cytotoxicity to cancer cells [3, 52]. Our group conjugated engineered HP-RNase 1 with trastuzumab as an IR in order to induce death in Her2 positive cell lines [37]. A cytotoxicity dose response assay revealed that this immunotoxin could induce death in different types of Her2 positive cell lines at an IC50 of 250 nM. It was concluded that the engineered enzyme had evaded the cytoplasmic RI of Her2 positive cell lines [37]. The scFv of trastuzumab with HP-RNase 1 was more potent than the trastuzumab alone due to the smart release and delivery to the three HER2 over-expressing breast cancer cell lines and herceptin-resistant cell line. These results indicated that the engineered scFv-Fc-HPR antibodies could be a promising human anti-cancer agent for breast

cancer therapy [37]. We also designed a new immunotoxin based on ranpirnase [54]. We engineered *Rana pipiens* RNase with 5 mutations (M23L, L45R, L49R, L55R and E91A) to bind to the heavy chain of human anti-epidermal growth factor receptor antibody. The molecular dynamic simulations confirmed protein stability and the ability of ranpirnase to bind to the epidermal growth factor receptor. The immunotoxin function was assessed in A431 cancer cells and HEK293 normal cells, and the IC_{50} was estimated at 39.50 and >2559 nM, respectively. The results indicated that the immunotoxins produced in this study against the EGFR receptor could be used as anticancer drugs [54].

A strong synergism was reported by Mikulski et al. [55] when ONC was combined with tamoxifen, trifluoroperazine (stelazine), or lovastatin to treat pulmonary carcinoma A549 or pancreatic adenocarcinoma ASPC-1 cells. Other studies also revealed a synergism of ONC with agents such as vincristine [56], interferons [57], differentiation-inducing agents [58], tumour necrosis factor α [59], cepharanthine [60], ionizing radiation [61], and, in vivo, with tamoxifen [62].

In 2006, Alfacell released interim data from the company's ongoing Phase IIIb randomized clinical trial of ranpirnase and doxorubicin for the treatment of malignant mesothelioma [63].The first interim analysis found 105 patient deaths of the total 316 patients enrolled. The results also indicated that the overall median survival time was 12 months for the ranpirnase plus doxorubicin treatment group and 10 months for the doxorubicin group. The immunoRNases have been under investigation in several malignant diseases, both in vitro and in animal models [64, 65].

Research is continuing in clinical trials on human pancreatic-type ribonuclease (QBI-139) with toxicity for cancer cells (Rains et al., data unpublished) to evaluate the toxicity, tolerability, and maximum tolerated dose of QBI-139 in patients with advanced and refractory solid tumours. Ontak (denileukin difitox) and lumoxiti have been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma and hairy cell leukemia, respectively [12, 21].

3.2. Monoclonal antibody

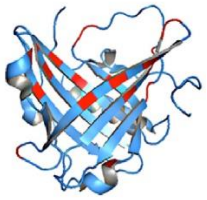
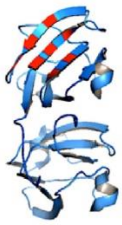
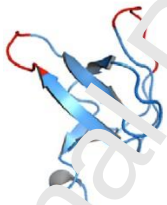
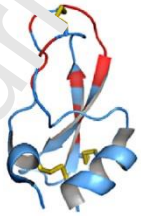
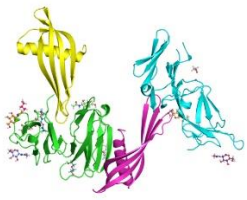
Antibodies are highly specific proteins that are composed of three fragments, including two identical antigen-binding sites (**Fabs**) and a constant region known as Fc. The variable domains located in the light and heavy chains of Fabs are involved in specific binding to the antigens [11, 66]. Research on the anti-tumour mechanism of the antibody proteins resulted in the development of monoclonal antibodies (**mAb**) that were approved by the FDA for

combination with other standard anti-cancer drugs [67, 68]. Various antibody-based molecules are undergoing preclinical and clinical trials including diabodies, multispecific, and multimodular antibodies [69, 70]. There are some limitations in the application of these antibody-derived therapeutics because of their large size, which decreases their penetration of the tumour cells, specifically tumours in solid tissues. Research has reduced the size of the mAb of the smaller variable fragments (**Fv**) in the Fabs regions. Antibodies with the smaller Fv regions have higher penetrating efficiency into solid tumorus than the native antibodies [70, 71].

3.3. New human protein scaffolds

To overcome some other challenges associated with the large size of mAbs, several researchers have concentrated on the production of small protein scaffolds (**Table 2**). According to the structural properties, the small non-antibody scaffolds are divided into two distinct classes -- domain sized scaffolds or peptide related types [72]. To date, one of the domain-sized scaffolds, known as Kunitz domain (kalbitor) was approved by the FDA for hereditary angioedema. Domain-sized scaffolds that have higher molecular weight, including anticalins, affilins, monobodies (**FN3**) and adnectins, were investigated [9, 27]. Clinical trials have proven that small non-immunoglobulin proteins have considerable potential as therapeutic and diagnostic tools, mainly because of their capacity for high specificity and target binding affinity. For example, several non-immunoglobulins, such as cyclotides (knottins), have been discovered in the last decade and approved for human use by the FDA. Anticalin, as another therapeutic protein scaffold, is currently being evaluated in clinical trials for tumour cell suppression.

Table 2. Structural features, molecular properties and specific targets of selected scaffold proteins.

Scaffold name	PDB code	Scaffold structure	Target protein	Structure	Molecular weight (kDa)
Anticalins	4GH7		Hepcidin IL-4Rα HGFR CD137/IL-17 Il-23/IL-17	β-sheet + α-helical terminus	20
Affilins	2JDF		Fibronectin EDB splice variant CTLA-4 VEGF-A	β-sheet α/β	20 10
FN3 (Adnectins)	1FNF		PCSK9 VEGFR2 Myostatin EGFR/IGF-1R	β-sheet	10
Fynomer	4AFS		TNF/IL-17A (FynomAb) HER2 (FynomAb)	β-sheet	7
Kunitz domains	4BQD		Kallikrein Neutrophil elastase Plasmin	α/β	7
Affimer	5MN2		VEGFR-2 Tenascin	α-helix + β-sheets	5

3.3.1. Anticalins

Anticalins, identified as ligand-binding proteins, are derived from lipocalin scaffolds containing a non-glycosylated peptide chain. Anticalins have recently emerged as an adaptable receptor for small molecules. These scaffolds are released in body fluids as stable proteins that can target various epitopes on different proteins [73]. Protein scaffolds have a smaller size than the conventional antibodies, and this property has significantly improved their efficiency for tissue penetration by decreasing renal filtration (**Fig. 2**). The plasma half-life of anticalins can be increased by integration with peptides containing proline, alanine, and serine [73]. Anticalins have potential therapeutic application in the treatment of several human disorders via targeting the receptors and ligand binding sites, or directly binding to their ligands and inhibiting their interaction with related receptors. Anticalin-based drug candidates are at the preclinical and clinical development stages. The therapeutic potential of anticalins specific for the IL4-α receptor is currently being investigated for the treatment of asthma. Additionally, anti-VEGF anticalins can act as novel anti-cancer agents by inhibiting angiogenesis in solid tumours [74]. It has been shown that fluorescent dyes and radioactive labels can be chemically integrated with anticalins and applied for medical and therapeutic purposes, including providing molecular images that differentiate tumour and healthy tissues [75].

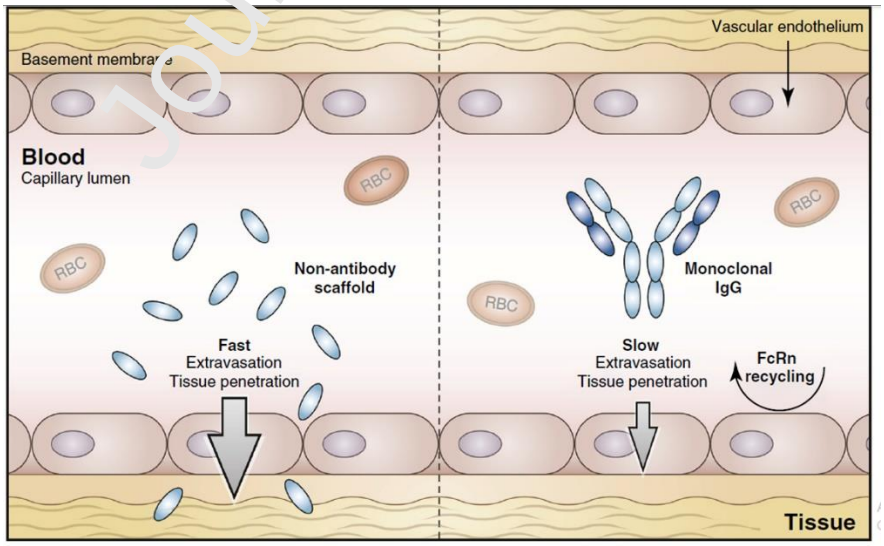


Fig. 2. Tissue penetration by scaffolds and monoclonal antibodies. The protein scaffolds have a high tissue penetration rate while monoclonal antibodies have a low tissue penetration.

Anticalins, as next-generation biologics for immuno-oncology, respiratory, and metabolic diseases, offer an alternative to antibodies, with promising and potentially superior features. The first anticalin protein to enter clinical study was selected against human vascular endothelial growth factor (**VEGF-A**) [76]. This anticalin protein (PRS-050) tightly binds VEGF-A and effectively prevents receptor binding and activation [77]. When PRS-050 was administered as a 2-h infusion at doses up to 10 mg/kg, it was well tolerated. No signs of toxicity or immunogenicity were observed. Significant reductions in circulating matrix metalloproteinase 2 (MMP-2) levels indicated an anti-angiogenic effect.

An anti-PCSK9 (proprotein convertase subtilisin/kexin type 9), another anticalin base fusion protein, showed convincing activity in the treatment of metabolic diseases such as dyslipidemia in preclinical research [78]. PCSK9 induces internalization and degradation by binding to a LDL receptor. Blocking the binding of PCSK9 to LDL-R by using an anti-PCSK9 antibody caused lower plasma levels of LDL cholesterol. Consequently, patients with atherosclerotic cardiovascular disease that received standard statin therapy had fewer cardiovascular events [78].

Anticalin fusion proteins also can be used as multispecific agents in immuno-oncology, particularly by addressing the ‘‘immunological synapse’’ that can form between a cancer cell and an immune cell at their interface [76]. Immunological synapse may increase activation of tumour-specific T cells near the tumour site, thereby avoiding some of the toxicities that are usually observed with peripheral T-cell activation in healthy tissues [78].

In order to treat respiratory diseases, such as asthma, an IL4-Rα-targeting anticalin protein, PRS-060, (AZD1402), is currently being developed jointly by Pieris and AstraZeneca as an inhalable biologic. This anticalin drug candidate is currently in a first-in-human phase I study in healthy volunteers, subject to clinical testing (NCT03384290, ClinicalTrials.gov).

A hepcidin-targeting anticalin fusion protein (PRS-080) was designed for the treatment of anemia [79]. Results from a phase I study in healthy volunteers showed that a single intravenous infusion of this protein (up to a dose of 16 mg/kg body weight) was safe and well tolerated. The safety of PRS-080 was verfied in patients with end-stage CKD requiring hemodialysis in a single-administration, and in a subsequent ascending-dose phase Ib study (up to 8 mg/kg body weight). Administration of this fusion protein caused a significant decrease in free hepcidin concentration within 1 h after infusion, with dose-proportional increases in both level and duration of serum iron concentration, as well as subsequent transferrin saturation [79]. In order to evaluate the safety and PK/ PD of repeated PRS-080

administration to anemic CKD patients undergoing hemodialysis, a phase IIa study was initiated (NCT03325621, ClinicalTrials.gov). Based on clinical studies on anticalins fusion protein and due to anticalins' versatility with regard to fusion protein generation and/or conjugation with drugs or radionuclides, anticalins appear to be a promising class of next-generation biopharmaceuticals.

3.3.2. Affilins

Affilin proteins are another class of affinity proteins that have various applications in therapy and purification. These scaffold proteins are generated from human γ-β crystallin from the human eye lens. The γ-β crystallin contains 176 amino acids that are folded into multiple b-sheet structures [80, 81]. These γ-β crystallin proteins have stability at high temperatures, a wide range of pH, and denaturing conditions that suggest their potential for therapeutic application. Recent findings reported that attachment of affilin molecules to the Fc fragment of IgG and also to fibronectin proteins that are upregulated in several tumours suggest that this affinity scaffold may be useful against various human malignancies [81]. Affilin proteins mainly express their function in both diagnostics and therapy by either changing signal transduction or by the delivery of designed components such as drugs or radionuclides [82].

Affilin ligands can be readily fused to the N - or C -terminus of an antibody's light or heavy chain, converting it into a bispecific format (mabfilin). For example, HER2-specific affilin was fused C-terminally to cetuximab (an EGFR-specific monoclonal antibody). This mabfilin was capable of binding to both HER2 and EGFR simultaneously [83]. The ease of creating multi-specific binding with affilin has potential in many areas of medicine. The application of multi-specific ligands for targeting cancer cells helps to circumvent problems such as antigen loss and tumour escape. The small molecules (8.5 kDa) make it possible to pack multiple affilin sequences into a lentiviral system for cell surface expression, offering greater flexibility within the limited genomic space [83]. In addition to therapeutic applications, these scaffolds can be used to create specifically tailored binding proteins as matrix-bound recognition tools in affinity chromatography [82].

3.3.3. Monobodies

The scaffold-based monobodies were originally derived from the human fibronectin type III domain (FN3). FN3-based monobodies (also called adnectins), as one of the well-studied fibronectin scaffolds, are composed of only one folded domain without any disulfide

bridges. The small structure of this type of antibody increases its efficiency for distribution to cancer cells and improves its stability in the intracellular cytosolic environment [84]. Adnectins are one of the therapeutic protein families that can bind to protein targets with high affinity and specificity, very similar to the machanism of antibody function [85]. Adnectin (CT-322) can suppress pancreatic cancer growth and metastasis through binding to vascular endothelial growth factor receptor 2 (VEGFR-2) and inhibiting the VEGF-A-induced signalling pathway [86, 87]. These scaffolds also have high tissue penetration and thermostability, which make them well suited for therapeutic applications [26]. Due to the high expression of fibronectin on cell surfaces, application of fibronectin scaffolds has less immunogenicity than other scaffold proteins; however, because of their small size, adnectins may be excreted by the kidneys and removed from the circulation. Therefore, their pharmacokinetic properties need to be improved by modern protein engineering approaches [88, 89].

Monobodies have another application related to adenoviruses (Ad). Ad are used as vectors for various types of functions. Nonspecific transduction of **Ad** vectors into cells or tissues after in vivo application may occur, which might lead to unexpected toxicity and tissue damage. A fiber-mutant Ad vector containing a monobody specific for epidermal growth factor receptor (EGFR) or vascular endothelial growth factor receptor 2 (VEGFR2) in the C-terminus of the knobless fiber protein derived from T4 phage fibritin was developed [90]. Surface plasmon resonance (SPR) analysis revealed that the monobody-displaying Ad vector specifically bound to the targeted molecules and led to significant increases in cellular binding and transduction efficiencies in the targeted cells. The results also indicated that transduction with the monobody-displaying Ad vectors was significantly inhibited in the presence of the Fc-chimera protein of EGFR and VEGFR2 [90]. Based on the results, it was concluded that this monobody-displaying Ad vector could be a crucial resource for targeted gene therapy.

Sullivan et al. [91] designed a novel strategy for deriving mimotopes to disease-specific serum antibodies. Anti-idiotypic monobodies were selected from a molecular library for identification of biomarkers of autoimmune disease to allow early diagnosis and initiation of treatment. The key feature of most autoimmune diseases is the production of autoantibodies, so much effort has focused on characterizing the antigens reactive with these antibodies. They evaluated this strategy by selecting a pool of serum immunoglobulins from a group of rheumatoid arthritis patients and evaluated selected clones for multi-patient reactivity and specificity [91]. This study indicated that use of the fibronectin scaffold to derive stable, easy to produce molecular probes for diagnosis of autoimmune disease could be of significant

value in improving diagnostic assays for virtually any disease that exhibits a characteristic immune response [91].

3.3.4. Affimer

Affimers are recombinant protein binders derived from two human protease inhibitors, stefin A and phytocystatin. Affimers can be expressed by bacterial sources, and they have high stability under different chemical conditions [92]. The structure of affimers consists of one α-helix and two pairs of β-sheet strands [93]. The scaffold binding sites are located between the β-sheets pairs and are made of two variable loops containing nine amino acids that can be replaced with other sequences. This enables affimers to have higher affinity and specificity for various target proteins. Having such properties makes affimers potentially suitable for bio-imaging and diagnostic applications [94]. Affimer protein technology has the potential to be applied to a variety of biological systems where speed, protein stability, and high yields are paramount. The key advantage of this technology is that affimer proteins can be expressed in cells, which provides a platform to assess intracellular function with the potential for development of therapeutic targets. Tiede et al. [95] demonstrated that affimer proteins can be generated against various target molecules that are useful in a plethora of biomedical applications.

The first affimer-sensor for the detection and estimation of the cancer biomarker Her4 in undiluted serum was developed [96]. Electrochemical impedance spectroscopy (EIS) results from this study demonstrated that the affimer sensor in buffer and in undiluted serum has high sensitivity with a broad dynamic range from 1.0 pM to 100 nM and a limit of detection lower than 1.0 pM both in buffer and in serum. Moreover, the affimer sensor demonstrated excellent specificity with negligible interference from serum proteins, suggesting resistance to non-specific binding [96]. The ability of this affimer sensor in spiked undiluted serum suggests its potential for a new range of affimer-based sensors. The fabricated affimer sensor can be adapted with other probes having affinities to other biomarkers for a new range of biosensors. Another affimer-based impedimetric biosensor was designed for the detection of FGFR3, a promising biomarker for early diagnosis of bladder cancer [97]. Data from this study indicated that this sensor platform may not only provide an effective tool for bladder cancer surveillance, but also pave the way for designing a new analytical method for monitoring other protein biomarkers of disease [97]. In vitro and in vivo studies have demonstrated that affimer proteins block the vascular endothelial growth factor-2

receptor (VEGFR2), a key regulator of vascular physiology including vasculogenesis, tumour neovascularization, and angiogenesis [95]. Xie et al. [98] developed an affimer-antibody immunological diagnosis kit for glypican-3, a serological and immunochemical marker for hepatocellular carcinoma. This biomarker may be a promising candidate for the detection of small and early-stage hepatocellular carcinoma. Advanced next-generation imaging, diagnostics, and therapeutics (theranostics) are now being developed in the form of engineered protein scaffold affimers, which are promising alternatives to antibodies (**Fig. 3**).

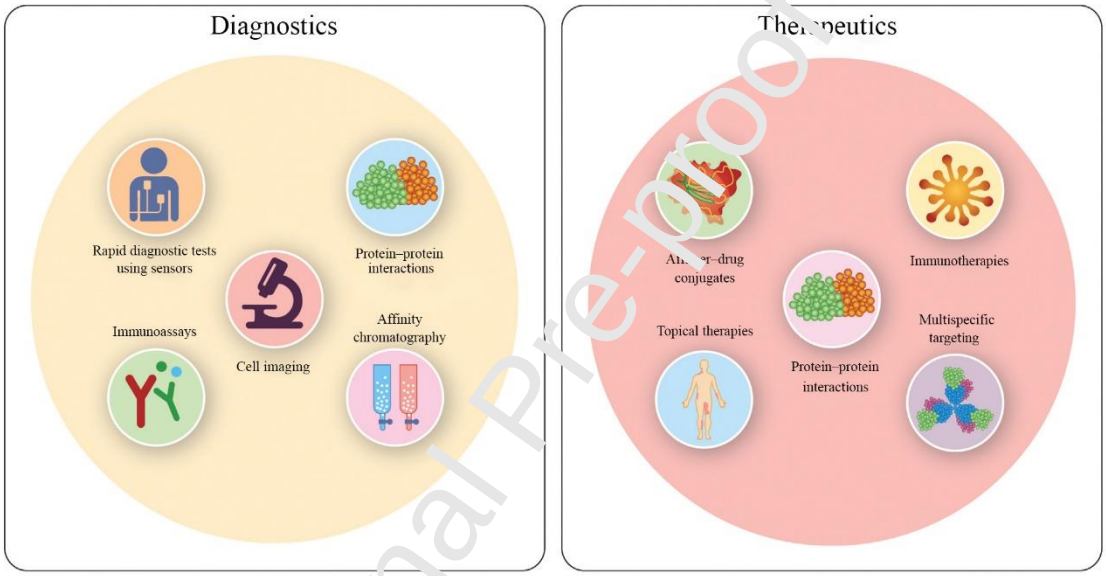


Fig. 3. Diverse imaging, therapeutic, and diagnostic (theranostic) applications of affimer protein binders.

3.3.5. Centyrin

Centyrins originated from fibronectin type III (FN3) domains of human tenascin. The scaffold contains seven beta-stranded sheets and connecting loops that potentially can be engineered for binding to different proteins in order to develop therapeutic proteins [99]. Centyrins can be expressed in *Escherichia coli*, and they have biophysical properties that include stability to heat and resistance to environmental pH. These properties help them to interact with a large number of molecules that make them suitable for advanced applications as a therapeutic scaffold. The amino acid sequence of centyrin consists of 100 residues without any cysteine and can be used for protein fusion applications [100, 101]. Because

centyrins are small, structurally simple, ultra-stable, and highly soluble proteins, they can be used for the discovery of medicines with new mechanisms of action against cancer and other devastating diseases. In addition, centyrins can be used to achieve cell-specific drug delivery to surface receptors and can form mono, bi or multi species in order to optimize binding and internalization of conjugated drugs such as oligonucleotides. A comprehensive engineering experiment was completed to find the best EGFR-binding centyrin [99]. All possible single cysteine centyrins were evaluated for expression, purification, conjugation efficiency, retention of target binding, biophysical properties and delivery of a cytotoxic small molecule. Twenty six of the 94 positions were identified as ideal for cysteine modification, conjugation and drug delivery. Conjugation-tolerant positions were mapped onto a crystal structure of the centyrin, providing a structural context for interpretation of the mutagenesis experiment and providing a foundation for a centyrin-targeted delivery platform.

3.3.6. Fynomer

Fynomers are small binding scaffold molecules derived from the SH3 domain of human FYN tyrosine kinase that can bind to various targets with high affinity and specificity. These scaffold proteins are composed of two β-sheets and two exposed loops for binding to target proteins [102]. The amino acid sequence of SH3 domains are very conserved from mouse to human and, therefore, regarded to be non-immunogenic for humans. These proteins have several properties that make them attractive for fusion applications. Fynomers have no cysteine residues and, hence, can be used for fusion purposes without any misfolding problem [102]. Fynomers can be fused to N-terminal and/or C-terminal ends of antibodies to generate bispecific therapeutics (Fynom Abs) with high-affinity binding domains to target proteins of interest and enhance activity, compared to the unmodified antibody [103]. Using phage display technology, fynomers binding to an epitope on HER2 were isolated that are different from the epitopes recognized by trastuzumab and pertuzumab [104]. Encouraging preclinical results indicate that the bispecific fynomer-antibody fusions have great potential for further preclinical and clinical development, both alone and in combination with trastuzumab [104].

Fynomers were used to form bispecific fusion proteins (FynomAbs) that can simultaneously bind HER2 on tumour cells and CD3 on T cells [105]. The bispecific HER2/CD3 targeting FynomAbs redirect T cells to HER2-expressing tumour cells, causing tumour cell lysis in multiple cell lines, using only picomolar quantities. The activity was found to be highly specific, as no lysis of cells was observed in the absence of HER2 expression. The FynomAbs also demonstrated an antibody-like pharmacokinetic profile in

mice, which may translate into a convenient administration route without the need for continuous infusion [105]. The fynomer 2C1 was engineered via phage display to bind the proinflammatory cytokine interleukin-17A (IL-17A) and was able to inhibit the activity of IL-17A in vitro with an IC50 of 2.2 nmol/L [102]. Fynomer 2C1 was subsequently fused to the Fc domain of a human antibody to prolong its circulation half-life. The resulting dimeric 2C1-Fc (Fc is a dimer) exhibited >100-fold improved IC50 against IL-17A (21 pmol/L) compared to the parent 2C1 molecule and effectively inhibited IL-17A in a mouse model of acute inflammation [102]. The researchers subsequently engineered FynomAb COVA322, a fusion molecule consisting of an IL-17A-binding fynomer fused to the anti-TNF antibody adalimumab. FynomAb COVA322 was designed to simultaneously inhibit the activity of both TNF and IL-17A for treatment of rheumatoid arthritis [106]. Bispecific FynomAb COVA322 inhibited IL-17A and TNF with in vitro IC50 values of 21 pmol/L and 169 pmol/L, respectively, and was effective in vivo [106]. COVA322 is currently being evaluated in a phase II clinical trial (NCT02243787). FynomAb represents an attractive platform to generate bispecific molecules and can be produced using standard antibody technology for selective killing of tumour cells.

3.3.7. Nanobodies

Nanobodies are small proteins produced from variable antigen-binding domains of antibodies. Nanobodies are produced by bacteria and yeast [107]. They can bind to target proteins with high affinity and specificity like monoclonal antibodies [108]. Due to their highly selective binding to target proteins, using nanobodies may have a lower risk for side-effects, which make them particularly well-suited for developing novel therapeutics. The nanobodies can bind to cell surface proteins like human epidermal growth factor receptor 2 (HER2) that have high expression in tumours. Nanobodies can regulate cancer cell growth and apoptosis by targeting HER2 proteins that are upregulated in breast and gastric cancer cells [109, 110]. Four nanobodies were evaluated in clinical trials for the treatment of cardiovascular and inflammatory disorders [111, 112]. The favorable properties of nanobodies such as high binding specificity, low toxicity and immunogenicity suggest that these affinity proteins may be introduced as highly effective next-generation therapeutic agents [113].

4. Conclusion

Therapeutic antibodies have led to a significant number of products used in the biopharmaceutical industry. However, from the functionality point of view, it is very

important to improve the quality of the antibody to achieve a reasonable balance between the apoptotic potency and the toxicity of the antibody. The RNase family, particularly immunoRNases, is highly effective for resisting and inhibiting tumour cells. Recent efforts to identify cancer-cell-specific antigens have been promising for designing novel immunoRNases that are capable of targeting tumour cells with high specificity and lower toxicity. The tumour cell is specifically targeted by the immunotoxin that is linked to both toxin and antibody. After entering into the cells, the conjugated part of the immunotoxin will selectively kill the tumour cells. However, the toxicity to normal cells and immunogenicity are two challenges to the clinical application of immunoRNases. The human RNases family segment of the immunoRNases can be replaced with a non-toxic engineered animal RNase. This provides a possibility for the animal RNase to act as an antiproliferation agent when interacting with the binding domain of the tumour cells' surface proteins without toxic and immunogenetic effects on healthy cells. Such a modification can provide an immunotherapeutic agent with higher toxicity, compared to the traditional methods using immunotoxins. For decades, the dominant modality for cancer chemotherapy has been small molecules developed using the tools of organic medicinal chemistry. However, in recent years, it has become clear that therapeutics based on biologically derived molecules such as monoclonal antibodies can have enormous benefits and advantages over small molecule approaches. Delivery of an external full monoclonal antibody with RNase (immunoRNase) to the cytosol of the tumour cells needs more efforts and investigation. Antibodies are ideally suited to target proteins that are located on the outside of cells, such as receptors; however, they are not readily taken into the cell (and contain disulfide bonds that are not stable inside the cell) and so are not a strong option for targeting proteins that carry out their normal function intracellularly. Production of full monoclonal antibodies as an immune part for immunoRNase is tedious and expensive. Therefore, we suggest substituting immunoRNases with small molecules such as monobody, affibody, anticalin, nonobody and affilin in future investigations. This will enhance the selectivity and specificity of killing tumour cells and can be an effective approach for cancer therapy, especially for treating solid cancers.

Achnowledgment

We would like to acknowledge the efforts of Prof. J. David Latshaw (The Ohio State University) for language editing of this article.

References

[1] Waks AG, Winer EP. 2019. Breast cancer treatment: a review. JAMA, 321:288-300, https://doi.org/10.1001/jama.2018.19323.

[2] De Lorenzo C, Arciello A, Cozzolino R, Palmer DB, Laccetti P, Piccoli R, D'Alessio G. 2004. A fully human antitumor immunoRNase selective for ErbB-2-positive carcinomas. Cancer Research, 64(14):4870-4, https://doi.org/10.1158/0008-5472.CAN-03-3717.

[3] Jordaan S, Akinrinmade OA, Nachreiner T, Cremer C, Naran K, Chetty S, Barth S. 2018. Updates in the development of immunoRNases for the selective killing of tumor cells. Biomedicines, 6(1):28, https://doi.org/10.3390/biomedicines6010028.

[4] Mathew M, Verma RS. 2009. Humanized immunotoxins: a new generation of immunotoxins for targeted cancer therapy. Cancer Science, 100(8):1359-65, https://doi.org/10.1111/j.1349-7006.2009.01192.x.

[5] Schirrmann T, Krauss J, Arndt MA, Rybak SM, Dübel S. 2009. Targeted therapeutic RNases (ImmunoRNases). Expert Opinion on Biological Therapy, 9(1):79-95, https://doi.org/10.1517/14712590802631862.

[6] Krauss J, Arndt MA, Dubel S, Rybak SM. 2008. Antibody-targeted RNase fusion proteins (immunoRNases) for cancer therapy. Current Pharmaceutical Biotechnology, 9(3):231-4,

[7] Stern LA, Case BA, Hackel BJ. 2013. Alternative non-antibody protein scaffolds for molecular imaging of cancer. Current Opinion in Chemical Engineering, 2(4):425-32, https://doi.org/10.1016/j.coche.2013.08.009.

[8] Dias AM, Roque AC. 2017. The future of protein scaffolds as affinity reagents for purification. Biotechnology and Bioengineering, 114(3):481-91, https://doi.org/10.1002/bit.26090.

[9] Schrama D, Reisfeld RA, Becker JC. 2006. Antibody targeted drugs as cancer therapeutics. Nature Reviews Drug Discovery. 5(2):147-59. https://doi.org/10.1038/nrd1957.

[10] Hantschel O. 2017. Monobodies as possible next-generation protein therapeutics–a perspective. Swiss Medical Weekly, 147(4748), https://doi.org/10.4414/smw.2017.14545.

[11] Allahyari H, Heidari S, Ghamgosha M, Saffarian P, Amani J. 2017. Immunotoxin: A new tool for cancer therapy. Tumor Biology, 39(2):1010428317692226, https://doi.org/10.1177/1010428317692226.

[12] Potala S, Sahoo SK, Verma RS. 2008. Targeted therapy of cancer using diphtheria toxin-derived immunotoxins. Drug Discovery Today, 13(17-18):807-15.

[13] Lorenzo CD, D'Alessio G. 2008. From immunotoxins to immunoRNases. Current Pharmaceutical Biotechnology, 9(3):210-214, https://doi.org/10.2174/138920108784567254

[14] Pastan I., Ho M. (2010) Recombinant Immunotoxins for Treating Cancer. In: Kontermann R., Dübel S. (eds) Antibody Engineering. Springer Protocols Handbooks. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-01147-4_10

[15] Chirino AJ, Ary ML, Marshall SA. 2004. Minimizing the immunogenicity of protein therapeutics. Drug Discovery Today, 9(2):82-90, https://doi.org/10.1016/S1359-6446(03)02953-2

[16] Bolhassani A, Talebi S, Anvar A. 2017. Endogenous and exogenous natural adjuvants for vaccine development. Mini Reviews in Medicinal Chemistry, 17(15):1442-56, https://doi.org/10.2174/1389557517666170228115801

[17] Choudhary S, Mathew M, Verma RS. 2011. Therapeutic potential of anticancer immunotoxins. Drug Discovery Today, 16(11-12):495-503, https://doi.org/10.1016/j.drudis.2011.04.003

[18] Kioi M, Husain SR, Croteau D, Kunwar S, Puri RK. 2006. Convection-enhanced delivery of interleukin-13 receptor-directed cytotoxin for malignant glioma therapy. Technology in Cancer Research & Treatment, 5(3):239-50, https://doi.org/10.1177/153303460600500307

[19] Chaudhary VK, Queen G, Junghans RP, Waldmann TA, FitzGerald DJ, Pastan I. 1989. A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin. Nature, 339(6223):394-7, https://doi.org/10.1038/339394a0

[20] Mei X, Chen J, Wang J, Zhu J. 2019. Immunotoxins: targeted toxin delivery for cancer therapy. Pharmaceutical Fronts, 1(1):e33-e45., https://doi.org/10.1055/s-0039-1700507

[21] Sahota S, Vahdat LT. 2017. Sacituzumab govitecan: an antibody–drug conjugate. Expert Opinion on Biological Therapy, 17(8):1027-31, https://doi.org/10.1080/14712598.2017.1331214

[22] Han L, Chen J, Ding K, Zong H, Xie Y, Jiang H, et al. 2017. Efficient generation of bispecific IgG antibodies by split intein mediated protein trans-splicing system. Scientific Reports, 7(1):1-11, https://doi.org/10.1038/s41598-017-08641-3

[23] Zewe M, Rybak SM, Dübel S, Coy JF, Welschof M, Newton DL, et al. 1997. Cloning and cytotoxicity of a human pancreatic RNase immunofusion. Immunotechnology, 3(2):127-36, https://doi.org/10.1016/S1380-2933(97)00070-5

[24] Iordanov MS, Ryabinina OP, Wong J, Dinh T-H, Newton DL, Rybak SM, et al. 2000. Molecular determinants of apoptosis induced by the cytotoxic ribonuclease onconase: evidence for cytotoxic mechanisms different from inhibition of protein synthesis. Cancer Research, 60(7):1983-94,

[25] Vazquez-Lombardi R, Phan TG, Zimmermann C, Lowe D, Jermutus L, Christ D. 2015. Challenges and opportunities for non-antibody scaffold drugs. Drug Discovery Today, 20(10):1271-83, https://doi.org/10.1016/j.drudis.2015.09.004

[26] Löfblom J, Frejd FY, Ståhl S. 2011. Non-immunoglobulin based protein scaffolds. Current Opinion in Biotechnology, 22(6):843-848, https://doi.org/10.1016/j.copbio.2011.06.002

[27] Taghizadegan N, Firozrai M, Jassiri M, Ariannejad H. 2020. Use of molecular dynamic tools in engineering of onconase enzyme to increase cellular uptake and evade RI. International Journal of Peptide Research and Therapeutics, 26:737-743, https://doi.org/10.1007/s10989-019-09881-9

[28] Weickmann JL, Elson M, Glitz DG. 1981. Purification and characterization of human pancreatic ribonuclease. Biochemistry, 20(5):1272-8, https://doi.org/10.1021/bi00508a035

[29] Psarras K, Ueda M, Yamamura T, Ozawa S, Kitajima M, Aiso S, et al. 1998. Human pancreatic RNase1-human epidermal growth factor fusion: an entirely human'immunotoxin analog'with cytotoxic properties against squamous cell carcinomas. Protein Engineering, 11(12):1285-1292, https://doi.org/10.1093/PROTEIN/11.12.1285

[30] Lima PO, Dos Santos FV, Oliveira DT, De Figueiredo RC, Pereira MC. 2015. Effect of eosinophil cationic protein on human oral squamous carcinoma cell viability. Molecular and Clinical Oncology, 3(2): 353-356, https://doi.org/10.3892/mco.2014.477

[31] Rosenberg H. 2008. Eosinophil-derived neurotoxin/RNase 2: connecting the past, the present and the future. Current Pharmaceutical Biotechnology, 9(3):135-40, https://doi.org/10.2174/138920108784567236

[32] Newton DL, Nicholls PJ, Rybak SM, Youle RJ. 1994. Expression and characterization of recombinant human eosinophil-derived neurotoxin and eosinophil-derived neurotoxin-anti-transferrin receptor sFv. Journal of Biological Chemistry, 269(43):26739-45, https://doi.org/10.1016/S0021-9258(18)47081-0

[33] Yoon JM, Han SH, Kown OB, Kim SH, Park MH, Kim BK. 1999. Cloning and cytotoxicity of fusion proteins of EGF and angiogenin. Life Sciences, 64(16): 1435–1445, https://doi.org/10.1016/S0024-3205(99)00077-6

[34] Li D, Bell J, Brown A, Berry CL. 1994. The observation of angiogenin and basic fibroblast growth factor gene expression in human colonic adenocarcinomas, gastric adenocarcinomas, and hepatocellular carcinomas. The Journal of Pathology, 172(2):171-5, https://doi.org/10.1002/PATH.1711720203

[35] Sun M, Sun L, Sun D, Zhang C, Li M. 2018. Targeted delivery of immuno-RNase may improve cancer therapy. Cancer Cell International, 18: 58, https://doi.org/10.1186/s12935-018-0546-7

[36] Laccetti P, Spalletti-Cernia D, Portella G, De Corato P, D'Alessio G, Giancarlo V. 1994. Seminal ribonuclease inhibits tumor growth and reduces the metastatic potential of lewis lung carcinoma. Cancer Research, 54(16): 4253-4256.

[37] Ghovvati, S. 2014. Engineering a ScFv-Fc Antibody-RNase Fusion Protein for HER2 Antigen Targeting. PhD Thesis, Ferdowsi University of Mashhad Mashhad.

[38] Sorrentino S. 2010. The eight human "canonical" ribonucleases: Molecular diversity, catalytic properties, and special biological actions of the enzyme proteins. FEBS Letters, 584(11):2194-200, https://doi.org/10.1016/j.febslet.2010.04.018

[39] Geng R, Liu H, Wang W. 2018. Differential expression of six RNase2 and three RNase3 paralogs identified in blunt snout bream in response to aeromonas hydrophila infection. Genes, 9(2):95, https://doi.org/10.3390/genes9020095

[40] Newton DL, Pollock D, Difiglio P, Echelard Y, Harvey M, Wilburn B, et al. 1999. Antitransferrin receptor antibody-RNase fusion protein expressed in the mammary gland of transgenic mice. Journal of Immunological Methods, 231(1-2):159-67, https://doi.org/10.1016/S0022-1759(99)00154-4

[41] Maeda T, Mahara K, Kita. oe M, Futami J, Takidani A, Kosaka M, et al. 2002. RNase 3 (ECP) is an extraordinarily stable protein among human pancreatic-type RNases. The Journal of Biochemistry, 132(5):737-42, https://doi.org/10.1093/oxfordjournals.jbchem.a003281

[42] Shimoyama S, Gansauge F, Gansauge S, Negri G, Oohara T, Beger HG. 1996. Increased angiogenin expression in pancreatic cancer is related to cancer aggressiveness. Cancer Research, 56(12):2703-6.

[43] Chopra V, Ding TV, Hanningan EV. 1997. Serum levels of interleukins, growth factors and anglogenin in patients with endometrial cancer. Journal of Cancer Research and Clinical Oncology, 123(3):167-72, https://doi.org/10.1007/BF01214669

[44] Ardelt W, Shogen K, Darzynkiewicz Z. 2008. Onconase and amphinase, the antitumor ribonucleases from Rana pipiens oocytes. Current Pharmaceutical Biotechnology, 9(3):215-25, PMID: 18673287

[45] Mikulski SM, Grossman AM, Carter PW, Shogen K, Costanzi JJ. 1993. Phase-I human clinical-trial of onconase (r)(p-30 protein) administered intravenously on a weekly schedule in cancer-patients with solid tumors. International Journal of Oncology, 3(1):57-64, https://doi.org/10.3892/IJO.3.1.57

[46] Mikulski S. 1999. Phase II trial of onconase in patients with advanced malignant mesothelioma: analysis of survival. Fifth International Meeting on Ribonucleases May 12-16, Warrenton, VA.

[47] Murthy BS, De Lorenzo C, Piccoli R, D'Alessio G, Sirdeshmukh R. 1996. Effects of protein RNase inhibitor and substrate on the quaternary structures of bovine seminal RNase. Biochemistry, 35(13):3880-5, https://doi.org/10.1021/bi952429m

[48] Sinatra F, Callari D, Viola M, Longombardo M, Patania M, Litrico L, et al. 2000. Bovine seminal RNase induces apoptosis in normal proliferating lymphocytes. International Journal of Clinical and Laboratory Research. 30(4):191-6, https://doi.org/10.1007/s005990070006

[49] Deonarain M, Epenetos A. 1998. Design, characterization and anti-tumour cytotoxicity of a panel of recombinant, mammalian ribonuclease-based immunotoxins. British Journal of Cancer, 77(4):537-46, https://doi.org/10.1038/bjc.1998.87

[50] Matousek J, Matousek J. 2010. Plant ribonucleases and nucleases as antiproliferative agens targeting human tumors growing in mice. Recent Patents on DNA & Gene Sequences, 4(1):29-39. https://doi.org/10.2174/187221510790410813

[51] Forouharmehr A, Nassiri M, Ghovvati Roudsari S, Jahadmanesh A. 2020. Production and introduction of a novel immunotoxin based on engineered RNase A for inducing death to Her1-positive cell lines. Journal of Cellular Physiology, 235(5):4675-87. https://doi.org/10.1002/jcp.29346

[52] Borriello M, Laccetti P, Terrazzano G, D'Alessio G, De Lorenzo, C. 2011. A novel fully human antitumour immunoRNase targeting ErbB2-positive tumours. British Jounal of Cancer, 104: 1716–1723, https://doi.org/10.1038/bjc.2011.146

[53] Riccio G, D'Avino C, Raines RT, De Lorenzo C. 2013. A novel fully human antitumor ImmunoRNase resistant to the RNase inhibitor. Protein Engineering, Design & Selection, 26: 243–248, https://doi.org/10.1093/protein/gzs101

[54] Ariannejhad H, Nassiri MR, Ghovvati G, Dehghani H, Asoodeh A. 2020. Designing of protein structure of ranpirnase as an immunotoxin based on bovine pancreatic ribonuclease using molecular dynamic and static simulations. Iranian Journal of Animal Science Research, 12: 351-360, https://doi.org/10.22067/ijasr.v12i3.81620

[55] Mikulski S, Viera A, Darzynkiewicz Z, Shogen K. 1992. Synergism between a novel amphibian oocyte ribonuclease and lovastatin in inducing cytostatic and cytotoxic effects in human lung and pancreatic carcinoma cell lines. British Journal of Cancer, 66: 304–310, https://doi.org/10.1038/bjc.1992.261

[56] Rybak SM, Pearson JW, Fogler W, Volker K. 1996. Enhancement of vincristine cytotoxicity in drug-resistant cells by simultaneous treatment with Onconase, an antitumor ribonuclease. Journal of the National Cancer Institute, 88: 747-53, https://doi.org/10.1093/JNCI/88.11.747

[57] Tang CHA, Hu CCA, Wei CW, Wang JJ. 2005. Synergism of Rana catesbeiana ribonuclease and IFN-γ triggers distinct death machineries in different human cancer cells. FEBS Letters, 579: 265-270, https://doi.org/10.1016/j.febslet.2004.11.086

[58] Halicka DH, Pozarowski P, Ita M, Ardelt WJ, Mikulski SM, Shogen K, Darzynkiewicz Z. 2002. Enhancement of activation-induced apoptosis of lymphocytes by the cytotoxic ribonuclease onconase (Ranpirnase). International Journal of Oncology, 21: 1245–1250, https://doi.org/10.3892/ijo.21.6.1245

[59] Deptala A, Halicka HD, Ardelt B, Ardelt W, Mikulski SM, Shogen K, Darzynkiewicz Z. 1998. Potentiation of tumor necrosis factor induced apoptosis by onconase. International Journal of Oncology, 13: 11-16, https://doi.org/10.3892/IJO.13.1.11

[60] Ita M, Halicka HD, Tanaka T, Kurose A, Ardelt B, Shogen K, Darzynkiewicz Z. 2008. Remarkable enhancement of cytotoxicity of onconase and cepharanthine when used in combination on various tumor cell lines. Cancer Biology & Therapy, 7: 1104-1108, https://doi.org/10.4161/cbt.7.7.6172

[61] Lee JE, Bae E, Bingman CA, Phillips GN, Raines RT. 2008. Structural basis for catalysis by onconase. Journal of Molecular Biology, 375: 165-177, https://doi.org/10.1016/j.jmb.2007.09.089

[62] Lee I, Lee YH, Mikulski SM, Shogen K. 2003. Effect of ONCONASE +/- tamoxifen on ASPC-1 human pancreatic tumors in nude mice. Advances in Experimental Medicine and Biology, 530: 187-96,

[63] Alfacell. 2006. Ranpirnase: amphibian ribonuclease A, P-30 protein-alfacell. Drugs in R & D, 8: 120-124,

[64] Lee I. 2008. Ranpirnase (Onconase), a cytotoxic amphibian ribonuclease, manipulates tumour physiological parameters as a selective killer and a potential enhancer for chemotherapy and radiation in cancer therapy. Expert Opinion on Biological Therapy, 8: 813-827, https://doi.org/10.1517/14712598.8.6.813

[65] Weber T, Mavratzas A, Kiesgen S, Haase S, Böttcher B, Exner E, Mier W, Grosse-Hovest L, Jäger D, Arndt MA, Krauss J. 2015. A humanized anti-CD22-onconase antibody-drug conjugate mediates highly potent destruction of targeted tumor cells. Journal of Immunology Research, 2015: 561814, http://dx.doi.org/10.1155/2015/561814

[66] Wozniak-Knopp G, Bartl S, Bauer A, Mostageer M, Woisetschläger M, Antes B, et al. Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. Protein Engineering, Design & Selection. 2010;23(4):289-97.

[67] Boyiadzis M, Foon KA. 2008. Approved monoclonal antibodies for cancer therapy. Expert Opinion on Biological Therapy, 8(8):1157-8,

[68] Zahavi D, Weiner L. 2020. Monoclonal antibodies in cancer therapy. Antibodies, 9(3):34, https://doi.org/10.3390/antib9030034

[69] Adams G, Weiner L. 2005. Monoclonal antibody therapy of cancer. Nature Biotechnology, 23: 1147–1157, https://doi.org/10.1038/nbt1137

[70] Todorovska A, Roovers RC, Dolezal O, Kortt AA, Hoogenboom HR, Hudson PJ. 2001. Design and application of diabodies, triabodies and tetrabodies for cancer targeting. Journal of Immunological Methods, 248(1-2):47-66, https://doi.org/10.1016/s0022-1759(00)00342-2

[71] Becker N, Benhar I. 2012. Antibody-based immunotoxins for the treatment of cancer. Antibodies, 1(1):39-69, https://doi.org/10.3390/antib1010039

[72] Binz HK, Amstutz P, Plückthun A. 2005. Engineering novel binding proteins from nonimmunoglobulin domains. Nature biotechnology. 23(10):1257-68, https://doi.org/10.1038/nbt1127

[73] Skerra A. 2008. Alternative binding proteins: Anticalins–harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities. The FEBS Journal, 275(11): 2677-83,

[74] Gebauer M, Skerra A. Engineered protein scaffolds as next-generation antibody therapeutics. Current Opinion in Chemical Biology, 13(3):245-55, https://doi.org/10.1146/annurev-pharmtox-010818-021118

[75] Skerra A. 2007. Anticalins as alternative binding proteins for therapeutic use. Current Opinion in Molecular Therapeutics. 9(4):336-44, PMID: 17694446

[76] Rothe C, Skerra A. 2018. Anticalin® proteins as therapeutic agents in human diseases. BioDrugs, 32: 233–243, https://doi.org/10.1007/s40259-018-0278-1

[77] Mross K, Richly H, Fischer R, Scharr D, Buchert M, Stem A, Gille H, Ausoly LP, Scheulen ME. 2013. First-in-human phase I study of PRS-050 (Angiocal), an anticalin targeting and antagonizing VEGF-A, in patients with advanced solid tumors. PLoS ONE 8(12): e83232, https://doi.org/10.1371/journal.pone.0083232

[78] Farnier M. 2014. PCSK9: From discovery to therapeutic applications. Archives of Cardiovascular Diseases, 107: 58-66, https://doi.org/10.1016/j.acvd.2013.10.007

[79] Renders L, Budde K, Rosenberger C, van Swelm R, Swinkels D, et al. 2019. First-in-human Phase I studies of PRS-080#22, a hepcidin antagonist, in healthy volunteers and patients with chronic kidney disease undergoing hemodialysis. PLOS ONE, 14(3): e0212023, https://doi.org/10.1371/journal.pone.0212023

[80] Jaenicke R, Slingsby C. 2001. Lens crystallins and their microbial homologs: structure, stability, and function. Critical Reviews in Biochemistry and Molecular Biology, 36(5):435-99, https://doi.org/10.1080/20014091074237

[81] Ebersbach H, Fiedler E, Scheuermann T, Fiedler M, Stubbs MT, Reimann C, et al. 2007. Affilin–novel binding molecules based on human γ-B-Crystallin, an all β-sheet protein. Journal of Molecular Biology, 372(1):172-85, https://doi.org/10.1016/j.jmb.2007.06.045

[82] Peltek OO, Muslimov AR, Zyuzin MV, Timin AS. 2019. Current outlook on radionuclide delivery systems: from design consideration to translation into clinics. Journal of Nanobiotechnology, 17(1):90, https://doi.org/10.1186/s12951-019-0524-9

[83] Kahl M, Settele F, Knick P, Haupts U, Bosse-Doenecke E. 2018. Mabfilin and Fabfilin - New antibody-scaffold fusion formats for multispecific targeting concepts. Protein Expression and Purification, 149: 51-65, https://doi.org/10.1016/j.pep.2018.04.013

[84] Chandler PG, Buckle AM. 2020. Development and differentiation in monobodies based on the fibronectin type 3 domain. Cells, 9(3):610, https://doi.org/10.3390/cells9030610

[85] Lipovšek D. 2011. Adnectins: engineered target-binding protein therapeutics. Protein Engineering, Design & Selection, 24(1-2):3-9, https://doi.org/10.1093/protein/gzq097

[86] Mamluk R, Carvajal IM, Morse BA, Wong HK, Abramowitz J, Aslanian S, et al. 2010. Anti-tumor effect of CT-322 as an adnectin inhibitor of vascular endothelial growth factor receptor-2. MAbs, 2(2):199-208, https://doi.org/10.4161/mabs.2.2.11304

[87] Dineen SP, Sullivan LA, Beck AW, Miller AF, Carbon JG, Mamluk R, et al. 2008. The Adnectin CT-322 is a novel VEGF receptor 2 inhibitor that decreases tumor burden in an orthotopic mouse model of pancreatic cancer. BMC Cancer, 8(1):352, https://doi.org/10.1186/1471-2407-8-352

[88] Ramamurthy V, Krystek Jr SR, Bush A, Wei A, Emanuel SL, Gupta RD, et al. 2012. Structures of adnectin/protein complexes reveal an expanded binding footprint. Structure, 20(2):259-69, https://doi.org/10.1016/j.str.2011.11.016

[89] Weidle UH, Auer J, Brinkmann U, Georges G, Tiefenthaler G. 2013. The emerging role of new protein scaffold-based agents for treatment of cancer. Cancer Genomics & Proteomics, 10(4):155-168.

[90] Matsui H, Sakurai F, Katayama K, Abe Y, Machitani M, Kurachi S, Tachibana M, Mizuguchi H. 2013. "A targeted adenovirus vector displaying a human fibronectin type III domain-based monobody in a fiber protein. Biomaterials, 34: 4191-4201, https://doi.org/10.1016/j.biomaterials.2013.02.046

[91] Sullivan M, Wentworth TC, Kobie J, Sanz I. 2012. Anti-idiotypic monobodies for immune response profiling. Methods, 58: 62-68, https://doi.org/10.1016/j.ymeth.2012.07.009

[92] Tans R, van Rijswijck DM, Davidson A, Hannam R, Ricketts B, Tack CJ, et al. 2020. Affimers as an alternative to antibodies for protein biomarker enrichment. Protein Expression and Purification, 105677, https://doi.org/10.1016/j.pep.2020.105677

[93] Kyle S. 2018. Affimer proteins: theranostics of the future? Trends in Biochemical Sciences, 43(4):230-2, https://doi.org/10.1016/j.tibs.2018.03.001

[94] Bedford R, Tiede C, Hughes R, Curd A, McPherson M, Peckham M, et al. 2017. Alternative reagents to antibodies in imaging applications. Biophysical Reviews. 9(4):299-308, https://doi.org/10.1007/s12551-017-0278-2

[95] Tiede C, Bedford R, Heseltine SJ, Smith G, Wijetunga I, Ross R, AlQallaf D, et al. 2017. Affimer proteins are versatile and renewable affinity reagents, eLife, 6:e24903, https://doi.org/10.7554/eLife.24903

[96] Zhurauski P, Arya SK, Jolly P, Tiede C, Tomlinson DC, Ferrigno PK, Estrela P. 2018 Sensitive and selective Affimer-functionalised interdigitated electrode based capacitive biosensor for Her4 protein tumour biomarker detection. Biosensors and Bioelectronics, 108: 1-8, https://doi.org/10.1016/j.bios.2018.02.041

[97] Thangsunan p, Lal N, Tiede C, Moul S, Robinson JI, Knowles MA, Stockley PG, et al. 2021. Affimer-based impedimetric biosensors for fibroblast growth factor receptor 3 (FGFR3): a novel tool for detection and surveillance of recurrent bladder cancer. Sensors and Actuators B: Chemical, 326: 128829, https://doi.org/10.1016/j.snb.2020.128829

[98] Xie C, Tiede C, Zhang X, Wang C, Li Z, Xu X, et al. 2017. Development of an Affimer-antibody combined immunological diagnosis kit for glypican-3. Scientific Reports, 7: 9608, https://doi.org/10.1038/s41598-017-10083-w

[99] Goldberg SD, Cardoso RM, Lin T, Spinka-Doms T, Klein D, Jacobs SA, et al. 2016. Engineering a targeted delivery platform using Centyrins. Protein Engineering, Design and Selection, 29(12):563-72, https://doi.org/10.1093/protein/gzw054

[100] Debie P, Hernot S. 2019. Emerging fluorescent molecular tracers to guide intra-operative surgical decision-making. Frontiers in Pharmacology, 10:510, https://doi.org/10.3389/fphar.2019.00510

[101] Chu F, Cao J, Neelalpu SS. 2018. Versatile CAR T-cells for cancer immunotherapy. Contemporary Oncology, 22(1A):73, https://doi.org/10.5114/wo.2018.73892

[102] Silacci M, Baenziger-Tobler N, Lembke W, Zha W, Batey S, Bertschinger J, et al. 2014. Linker length matters, Fynomer-Fc fusion with an optimized linker displaying picomolar IL-17A inhibition potency. Journal of Biological Chemistry, 289(20):14392-8, https://doi.org/10.1074/jbc.M113.534578

[103] Sedykh SE, Prinz VV, Buneva VN, Nevinsky GA. 2018. Bispecific antibodies: design, therapy, perspectives. Drug Design, Development and Therapy, 12:195-208, https://doi.org/10.2147/DDDT.S151282

[104] Mourlane F, Toller I, Brack S, Grabulovski D, Bertschinger-Ehrler J. 2012. Bispecific Fynomer-antibody fusion proteins targeting two epitopes on HER2. Journal of Clinical Oncology, 30: 2575-2575, https://doi.org/10.1200/jco.2012.30.15_suppl.2575

[105] Woods R, Brack S, Klupsch K, Wuellner U, Buller F, Santimaria R. 2014. Fynomer-antibody fusions targeting HER2 and CD3 for selective killing of HER2 overexpressing tumor cells. Journal of Clinical Oncology, 32: 3066-3066, https://doi.org/10.1200/jco.2014.32.15_suppl.3066

[106] Silacci M, Lembke W, Woods R, Attinger-Toller I, Baenziger-Tobler N, Batey S, Santimaria R, von der Bey U, Koenig-Friedrich S, Zha W et al. 2016. Discovery and characterization of COVA322, a clinical-stage bispecific TNF/IL-17A inhibitor for the treatment of inflammatory diseases. MAbs, 8 (1): 141-9.

[107] Steeland S, Vandenbroucke RE, Libert C. 2016. Nanobodies as therapeutics: big opportunities for small antibodies. Drug Discovery Today, 21(7):1076-113, https://doi.org/10.1016/j.drudis.2016.04.003

[108] Bannas P, Hambach J, Koch-Nolte F. 2017. Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. Frontiers in Immunology, 8:1603, https://doi.org/10.3389/fimmu.2017.01603

[109] Gray MA, Tao RN, DePorter SM, Spiegel DA, McNaughton BR. 2016. A nanobody activation immunotherapeutic that selectively destroys HER2-positive breast cancer cells. ChemBioChem, 17(2):155-8, https://doi.org/10.1002/cbic.201500591

[110] Arezumand R, Alibakhshi A, Ranjbari J, Ramazani A, Muyldermans S. 2017. Nanobodies as novel agents for targeting angiogenesis in solid cancers. Frontiers in Immunology, 8:1746, https://doi.org/10.3389/fimmu.2017.01746

[111] Revets H, De Baetselier P, Muyldermans S. 2005. Nanobodies as novel agents for cancer therapy. Expert Opinion on Biological Therapy, 5(1):111-24, https://doi.org/10.1517/14712598.5.1.111

[112] Bala G, Blykers A, Xavier C, Descamps B, Broisat A, Ghezzi C, et al. 2016. Targeting of vascular cell adhesion molecule-1 by 18F-labelled nanobodies for PET/CT imaging of inflamed atherosclerotic plaques. European Heart Journal-Cardiovascular Imaging, 17(9):1001-1008, https://doi.org/10.1093/ehjci/jev348

[113] Kolkman JA, Law DA. 2010. Nanobodies–from llamas to therapeutic proteins. Drug discovery today: Technologies, 7(2):e139-e46, https://doi.org/10.1016/j.ddtec.2010.03.002

CRediT author statement

Mohammadreza Nassiri, Reihane Behnam-Rasouli: Data curation, Writing-Original draft preparation. **Masoume Vakili-Azghandi**: Investigation, Writing. **Vinod Gopalan***:* Supervision. **Peyman Dolati**: Validation, Image Preparation. **Rouhollah Nourmohammadi:** Writing- Review and Editing,

Graphical abstract