Commentary

Defining the pathway to insulin-like growth factor system targeting in cancer☆

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A B S T R A C T

The insulin-like growth factors (IGFs; IGF-1 and IGF-2) play central roles in cell growth, differentiation, survival, transformation and metastasis. The biologic effects of the IGFs are mediated by the IGF-1 receptor (IGF-1R), a receptor tyrosine kinase with homology to the insulin receptor (IR). Dysregulation of the IGF system is well recognized as a key contributor to the progression of multiple cancers, with IGF-1R activation increasing the tumorigenic potential of breast, prostate, lung, colon and head and neck squamous cell carcinoma (HNSCC). Despite this relationship, targeting the IGF-1R has only recently undergone development as a molecular cancer therapeutic. As it has taken hold, we are witnessing a robust increase and interest in targeting the inhibition of IGF-1R signaling. This is accentuated by the list of over 30 drugs, including monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) that are under evaluation as single agents or in combination therapies [1]. The IGF-binding proteins (IGFBPs) represent the third component of the IGF system consisting of a class of six soluble secretory proteins. They represent a unique class of naturally occurring IGF-antagonists that bind to and sequester IGF-1 and IGF-2, inhibiting their access to the IGF-1R. Due to their dual targeting of the IGFs without affecting insulin action, the IGFBPs are an untapped "third" class of IGF-1R inhibitors. In this commentary, we highlight some of the significant aspects of and prospects for targeting the IGF-1R and describe what the future may hold.

1. Introduction

The IGF system is comprised of the IGF-1R, IGF-1, IGF-2, insulin and six soluble IGF-binding proteins (IGFBP-1-6; Fig. 1a). The IGF-1R (and insulin receptor; IR) is a type 1 receptor tyrosine kinase (RTK) initially synthesized as a single chain precursor that is cleaved and disulfide bonded within the Golgi complex to yield a

heterodimer and finally a heterotetramer. The immediate downstream target of the IGF-1R tyrosine kinase is the insulin receptor substrate protein (IRS-1; [2]), which uniquely serves as a scaffold for binding downstream targets of the IGF-1R. Once IRS-1 (or IRS-2) becomes tyrosine phosphorylated, effectors are recruited and bind via their SH2 or PTB domains to mediate IGF-1R actions. This differs from other receptor tyrosine kinases (RTKs), which directly bind to their immediate downstream effectors. There are exceptions to this modality with signaling to extracellular signal regulated kinases (Erks 1/2) triggered by the binding of Src homologous and collagen containing (Shc) signaling protein, Shc-66, to the IGF-1R [3].

The IGFBPs consist of a unique class of proteins capable of binding IGF-1 and IGF-2 with higher affinity than their interactions with the IGF-1R, but do not bind to insulin. This sequestration of IGF-1 and IGF-2 results in reduced IGF-1R signaling. It is likely that such fine-tuning of IGF-1R action provided a distinct evolutionary advantage that is unique to the IGF system. The role of the IGF system in normal physiology, development, aging, pathology and cancer provide a rationale in support of such tight control. This, in turn, begs the question "how did the IGFBPs evolve their own IGF-independent actions", many of which run counter to the effects of inhibiting IGF-1R signaling [4]. The IGFBPs appear to lack cell surface receptors for regulating IGF-independent cellular

functions. In this regard, IGFBP-3 and IGFBP-5 are most similar to one another and each have within their structures, a peptide stretch that is cell membrane permeable [5] providing a mechanism whereby these proteins may enter cells to elicit IGF-independent effects. In that context, IGFBP-3 has had the most IGF-independent actions and sites of actions described [6].

Signaling by the IGF-1R and its dysregulation has been noted to be contributory to a variety of diseases including, diabetic retinopathy [7], diabetic nephropathy [8], age-related macular degeneration [9], cardiovascular disease, aging and in a variety of cancers [10]. Due to space considerations, we will focus our discussion on cancer and review the current state of IGF-1R targeting by mAb and TKI approaches. We will then make the case for considering IGFBP-2 as a tractable inhibitor of IGF-1R signaling

for clinical use given its ability to block both IGF ligands and to potentially lack the side effects and toxicities common to the current inhibitory drugs.

1.1. The IGF system in cancer

It is generally accepted that the growth-promoting activities of IGF-1, IGF-2 and insulin are mediated by the IGF-1R and that the IGF-2R serves as a clearance receptor, removing IGF-2 from the cell surface. Elevated IGF-1R expression and activity has been associated with multiple aspects of cancer progression including enhanced carcinogenesis, tumorigenesis, metastasis, resistance to chemotherapeutics and other molecularly targeted drugs and to transformation [11]. In addition to the aforementioned IGF system
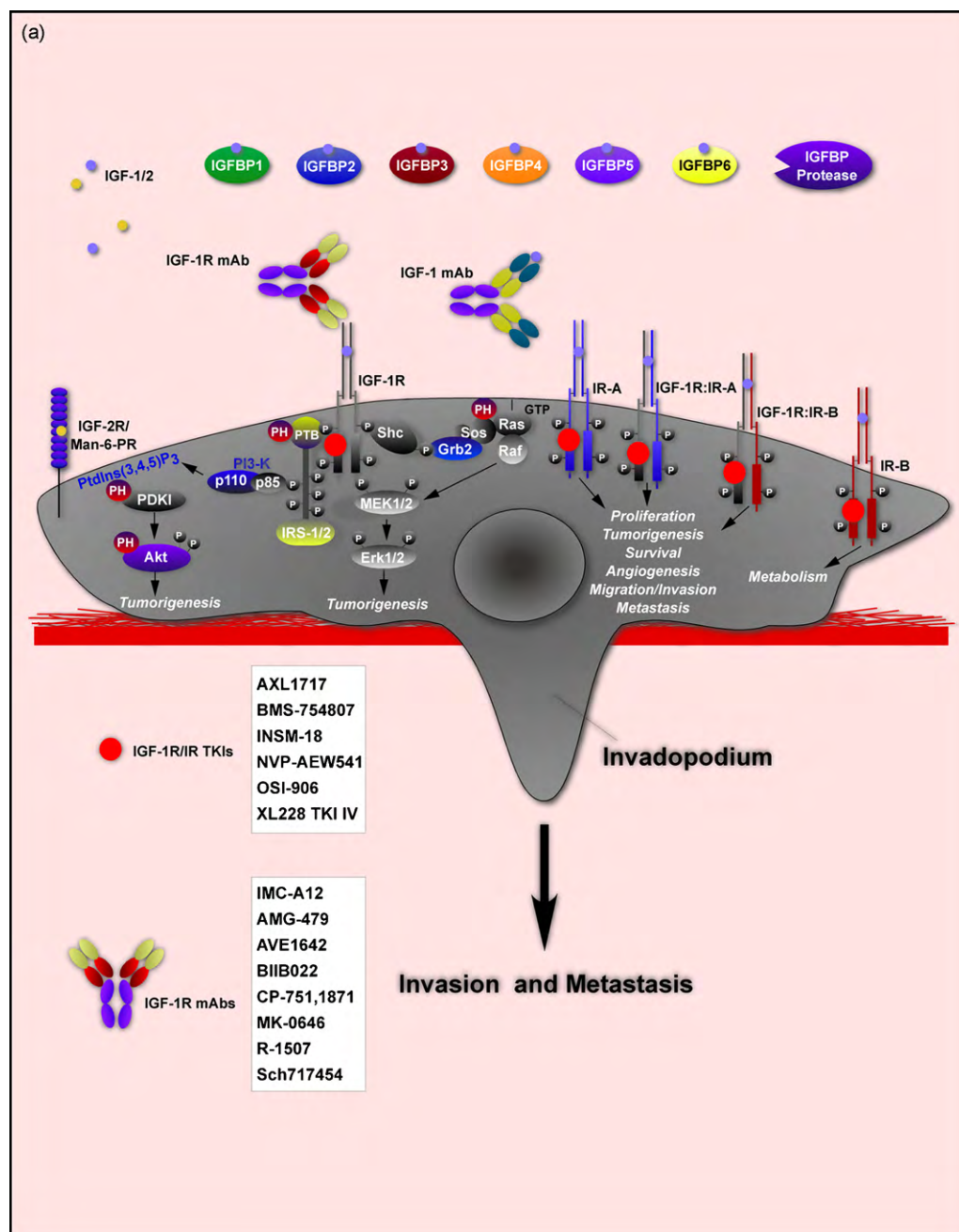
Fig. 1. The IGF system in cancer. (a) *IGF system components*. Shown are the ligands, IGF-1/IGF-2, the receptors, IGF-1R, IR-A, IR-B and hybrid IGF-1R:IR-A and IGF-1R:IR-B receptors and IGFBPs. The mAbs and TKIs targeting the IGF-1R is an incomplete list. (b) *Targeting the tumor microenvironment IGF system*. Within the tumor microenvironment, multiple cell types secrete IGF-1 that diffuses to tumor cells to enhance tumorigenesis. IGFBP inhibition of IGF-1 and IGF-2 is well suited for the tumor microenvironment where multiple sources of IGF-1 exist. Also shown is increased angiogenesis mediated by VEGF downstream of IGF-1R action [99,100,102].
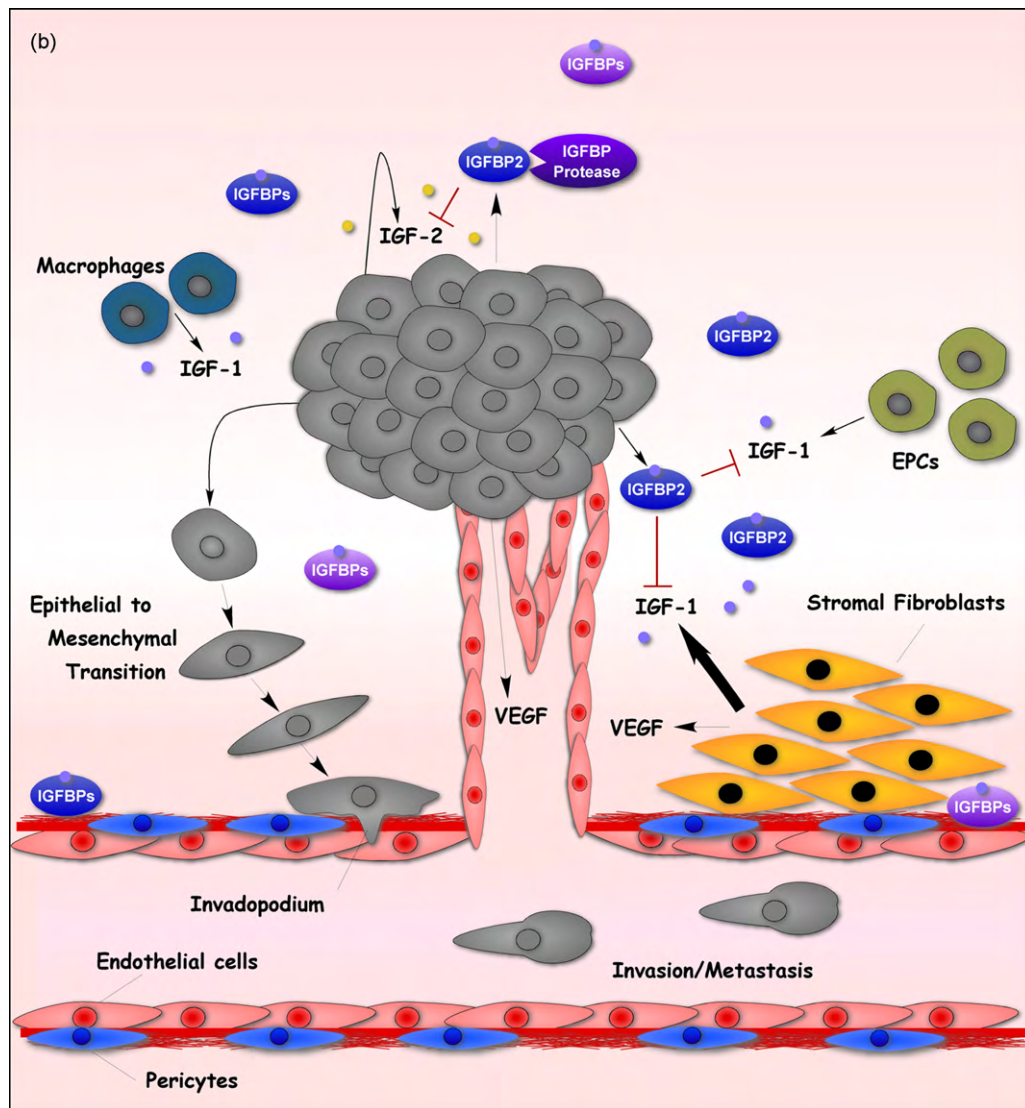
Fig. 1. (*Continued*).

components, two isoforms of the IR exist, IR-A and IR-B resulting from the alternative splicing of the 22 exon human IR gene [12]. As a result of skipping exon 11 (36 bp) during development and tissue-specific expression, IR-A differs from IR-B by a 12 amino acid truncation (residues 717–728) at the C-terminal end of the α-subunit. While IR-A is widely distributed across tissues, IR-B is present in liver, muscle, adipose and kidney and regulates metabolism and glucose uptake [12]. IR-A is expressed in fetal tissues and in cancer cells, preferentially binds to IGF-2 and regulates growth-promoting actions [13,14]. Hybrid receptors consisting of IGF-1R:IR-A or IGF-1R:IR-B heterotetramers bind to IGF-2 or insulin and IGF-1, respectively, and participate in cancer cell signaling paradigms [15]. It is in this context that the ability of IGF-1R TKIs to inhibit both the IGF-1R and IR or dual specificity IGFBPs (blocking IGF-1 and IGF-2) would be most beneficial.

An important clue to the essential role of the IGF-1R in cell function was uncovered by Baserga and coworkers [16] who reported that IGF-I signaling was an absolute requirement for viral transformation of cells. These findings and subsequent analyses revealed that many oncogenes require IGF-1R signaling to be effective [2,17,18]. This is consistent with the well studied pro-survival signaling properties of the IGF-1R mediated by Akt [19]. Engagement of this signaling pathway increases the propensity of

cells harboring cancerous mutations to survive rather than undergo apoptosis.

The growth-promoting effects of the IGF-1R are related to the permissive nature of IGF-1R signaling, which supports the microenvironment in a manner that can enhance tumorigenesis. The autocrine and paracrine functions of its two principal ligands appear to be dysregulated in cancer. IGF-2 is imprinted and only expressed from the paternal allele. When imprinting is lost the result is IGF-2 overexpression [20]. The IGF-2 gene is the most overexpressed gene in colorectal cancer [21] consistent with signaling by this ligand being capable of enhancing tumorigenesis [17] including β-cell tumorigenesis [22]. Baserga and coworkers [16] were the first to demonstrate that oncogenic transformation of cells required functional IGF-1Rs, underscoring the importance of autocrine and paracrine IGF-2 and IGF-1 in tumors and the tumor microenvironment, respectively, in supporting tumorigenic progression. An example of the tight regulation of these pathways by the IGFBPs is evident from studies on colonic myofibroblasts where MMP-7 cleavage of IGFBP-5 releases bound IGF-2 which then acts as a myofibroblast mitogen [23].

It has been pointed out that the IGF-1R alone does not mediate growth and transforming activities, but rather the pathway itself, which is administered by IRS-1, signals to growth promoting and

anti-apoptotic pathways [2]. IRS-1 has 18 potential sites of tyrosine phosphorylation that serve as SH2 domains for docking downstream effectors; constitutively phosphorylated IRS-1 has been found in a number of cancers [24]. This has led to the hypothesis that IRS-1 may be the preferred target for cancer therapeutics, given that it is regulated by IGF-1R, IR, cytokine receptors [25] and EGFRs [11]. It is clear that IRS-1 is a key hub overseeing downstream signaling actions of the IGF-1R. Consistent with its central role in survival signaling, Baserga has referred to IRS-1 as an anti-tumor suppressor acting as an anti-p53 protein [18]. In cancer, there are numerous alterations in signaling pathways that modify the standard signaling nodes. For example, breast cancer cell resistance to estrogen deprivation results in alternative signaling pathways and association of estrogen receptor-α with Shc, Src, EGFR and the IGF-1R [26]. Recent studies on IGF-1R and EGFR signal crosstalk revealed that phosphoinositide-dependent kinase 1 (PDK1) is tyrosine phosphorylated by and binds directly to the IGF-1R [27].

2. Targeting the IGF-1R: early reluctance of this strategy

While a number of non-receptor tyrosine kinases (non-RTKs) and RTKs have been targeted since FDA approval of the HER2/neu inhibitory monoclonal antibody (mAb) trastuzumab (Herceptin®) in 1998 and the Bcr-Abl tyrosine kinase inhibitor (TKI) imatinib (Gleevec®) in 2001, targeting the IGF-1R has been slow to catch on. This reluctance has largely been due to concerns that inhibition of this key system that is so crucial to normal physiology might have too many side effects and toxicities. This engendered a view that targeting the IGF-1R was an unattractive proposition [28] or at the very least, a delicate balancing act [29]. This primarily stems from the well known ubiquitous distribution of IGF-1Rs in normal tissues [30] and the inhibition of IR signaling exhibited by IGF-1R directed RTKIs. Given that excellent specificity has been retained for TKIs and notwithstanding the fact that tyrosine kinase domains of RTKs and non-RTKs share upwards of 90% sequence homology and identity, targeting the IGF-1R tyrosine kinase domain has become an active area of research, as has the development of IGF-1R targeting monoclonal antibodies [1].

2.1. Why the IGF-1R and why now?

The IGF-1R is an essential regulator of transformation, pro-survival anti-apoptotic signaling and is known to have a role in the resistance to chemotherapeutic and radiation therapies, all of which serve to underscore the attractiveness of its targeting as a means of killing tumor cells. Yet, targeting IGF-1R had been of less interest despite its well known role in contributing to the cancer cell autocrine growth regulation as the second hallmark of cancer and its role in invasion and metastasis, the sixth hallmark [31]. The principal reason was concern that IGF-1R TKIs would also target the IR as these receptors share ∼60% overall sequence identity and ∼80% kinase domain sequence identity [32]. Accordingly, one would predict that inhibition of the IGF-1R using a TKI would likely inhibit IR signaling, resulting in a diabetogenic state characterized by hyperglycemia. Indeed, hyperglycemia is the primary adverse effect of the IGF-1R TKIs currently being evaluated in clinical trials. It has been treated by administering metformin [33]. This introduces an additional rationale for why there should be increased interest in targeting the IGF system. Despite their expression in multiple cancer types, IGF-1Rs were overlooked as viable targets due to their lack of amplification/mutation, unlike other receptors, such as HER2/neu, which exhibits overexpression and ligand-independent activation as a result of gene amplification [34].

An additional aspect relevant to the targeting of IGF-1Rs is the mounting link between diabetes and cancer incidence as described

for colon, pancreatic and post-menopausal breast cancer [35]. Moreover, recent epidemiologic studies have raised concern over the use of long-acting insulin (glargine insulin, Lantus®, Sanofi-Aventis) based on its potential to increase cancer incidence [36]. There are two aspects to consider: (1) will IGF-1R TKI therapy result in hyperglycemia and a diabetic state and; (2) does having type 2 diabetes predispose patients to being more cancer-prone [36]. A recent study in Germany comparing diabetic patients taking human insulin, short acting analogs or long-acting glargine insulin revealed a greater than expected increase in cancer incidence in the glargine group compared to those taking human insulin [37]. Related to the diabetes and cancer connection, Goodwin et al. [38] reported that high levels of fasting insulin led to poor breast cancer outcomes and that these women were candidates for new and more effective treatment strategies. Here is where the use of alternative drug species, such as IGFBP-2 may provide a benefit. IGFBP-2 is the second most abundant IGFBP in the circulation after IGFBP-3. Its levels are relatively stable and unaffected by meals or glucose levels [39] with serum IGFBP-2 levels being inversely proportional to insulin levels; IGFBP-2 transgenic mouse studies have revealed minimal adverse effects (reviewed in [40], see Section 11 below).

3. Ready, aim, fire: the IGF-1R is a target

Despite the many barriers to targeting the IGF-1R, numerous pharmaceutical and biotechnology companies have developed molecularly targeted reagents against this receptor, primarily employing mAb and TKI approaches (Fig. 1; Table 1; reviewed in [1,33]). One of the common occurrences seen with mAb and TKI therapies directed against RTKs is toxicity. A case in point for mAbs is trastuzumab (Herceptin), which is associated with congestive heart failure [41], likely the result of targeted receptors being present on cardiac myocytes. The issue of receptor localization also holds true for TKIs as does the fact that these small molecules gain access to the large set of intracellular proteins with which they interact and modify functionally, consistent with their additional toxicities and side effects [42]. Such generalized toxicities have been observed in the early testing of IGF-1R targeted monoclonal antibodies and RTKIs [17,42] leading to considerable disappointment. This has occurred despite the high targeting/receptor specificity of these agents [1,33]. The precise mechanisms responsible for these negative outcomes are currently unclear. It is because of these confounding effects, alternate means of inhibiting this receptor should be considered, including the use of the IGFBPs.

There are currently an estimated 30 drugs in various stages of development that target IGF-1R signaling. Of those targeting the IGF-1R, approximately half are receptor directed mAbs and the other half are TKIs. These represent two distinct classes of drugs of the standard four categories of cancer therapeutics which include (1) small molecule inhibitors, (2) mAbs, (3) natural products from plants and (4) natural products from microorganisms [43], each of which have their pros and cons with respect to selectivity/specificity, toxicities and probability of inducing resistance to long term therapy.

4. IGF system targeting strategies: IGFBPs are a novel class of natural product IGF-antagonists

The prevalence of toxicities to IGF-1R directed mAbs and TKIs begs the question of whether targeting the ligands, IGF-1 and IGF-2 might be a viable alternative with the potential of reduced toxicities other than hyperglycemia. A successful ligand-targeting strategy comes from the vascular endothelial growth factor (VEGF) targeting mAbs, bevacizumab (avastin) and ranibizumab (lucentis [44]). To date, there has been limited development of IGF-1 or IGF-

Table 1
Drugs targeting the IGF system.

Inhibitor	Isotype	Company
(A) Anti-IGF-1R mAbs		
IMC-A12	Fully human IgG1 (cixutumumab)	Imclone, Inc., NY, NY
AMG-479	Fully human IgG1	Amgen, Thousand Oaks, CA
AVE1642	Humanized IgG1	Sanofi-Aventis, Paris, France
BIIB022	Human non-glycosylated IgG4	Biogen, Cambridge, MA
CP-751,1871	Fully human IgG2 (figitumumab)	Pfizer, NY, NY
MK-0646	Humanized IgG1	Merck, Whitehouse Station, NJ
R-1507	Fully human IgG1 (robatumumab)	Roche, Basel, Switzerland
Sch717454	Fully human IgG1 (19D12)	Schering-Plough, Kenilworth, NJ

Inhibitor	Company
(B) IGF-1R TKIs	
AXL1717	Axelar, Stockholm, Sweden (picropodophyllin)
BMS-754807	Bristol-Myers Squibb, Princeton, NJ
INSM-18	Insmed, Richmond, VA; IGF-1R and HER2/neu inhibitor
OSI-906	OSI Pharmaceuticals, Melville, NY
NVP-AEW541	Novartis, Basel, Switzerland
XL228 TKI IV	Exelixis, South San Francisco, CA (IGF-1R, Src, FGFR, Bcr-Abl)

IGF-1R anti-sense oligos/siRNA	Company
ATL1101	Antisense Therapeutics, Ltd.

Ligand sequestration strategy	Reference
(C) IGF-1 and IGF-2	
rhIGFBP-3 (mecasermin)	Insmed, Inc., Richmond, VA (formerly Celtrix)
IGFBP-1	Yee [103,104]
IGFBP-2	Horney et al. [8]; Kibbey et al. [76]
IGFBP-mimetics	Sidhu and coworkers [81]; Clemmons and coworkers [105]; Robinson and Rosenzweig [106]
Anti-IGF-1/IGF-2 antibodies	IGF-1/IGF-2 [107]; IGF-2 [108]
Soluble IGF-1R (decoy)	IGF-1R 486 stop [109,110]

2 directed antibody therapeutics (Table 1). Along these lines, the IGFBPs are viable alternatives to mAbs in ligand targeting, with the added advantages that they bind to both IGF-1 and IGF-2 and are natural products. Although they are natural products, it is largely unknown whether toxicities would be associated with their therapeutic use, unlike the use of mAbs and receptor tyrosine kinase inhibitors [42]. To date, the only IGFBP applied therapeutically is IGFBP-3, which is available for clinical use both as a single agent (mecasermin) and in a complex with IGF-1 as mecasermin rinfabate or iPlex™ [45]. The formulated complex is designed to reduce the adverse affects of IGF-1 treatment, such as hypoglycemia. The application and proof of concept of introducing mutations into natural proteins for the development of novel, more effective protein-based therapeutics is already well developed with the introduction of shorter acting (lispro insulin) and longer acting insulin molecules (glargine [46], see Section 6 below).

5. IGFBPs as cancer chemopreventive agents

It is worth mentioning a chemoprevention approach to therapeutics, given that many agents have the potential of up-regulating the IGFBPs. Vitamin D increases IGFBP-3 expression [47] and has been under investigation for use in colorectal [48] and prostate cancers [49]. The tumor suppressor p53 induces IGFBP-3 expression [50] providing insight into one of the multiple ways p53

blocks cell growth. Retinoids induce IGFBP-5 [51] and IGFBP-3 [17,52] as do antiestrogens and TGF-β [53], the flavonoid silibinin from milk thistle [54], the green tea flavonoid, epigallocatechin gallate EGCG [55], and grape seed extract [56]. On the negative side of this approach, IGFBP-2 was shown to be downstream of the PI3K/Akt pathway, with loss of function PTEN mutants increasing IGFBP-2 in glioblastoma and prostate cancer and correlating with a poor prognosis [57]. The opposite preventive approach to up-regulating IGFBP levels is to block their proteolysis by the administration of proteinase inhibitors. An example of the therapeutic use of a proteinase inhibitor is the oral hypoglycemic agent sitagliptin. It is a dipeptidyl peptidase-IV inhibitor that raises the level of circulating incretin by reducing its proteolysis; it is administered either as a monotherapy or in combination with insulin and/or metformin in type 2 diabetics [58].

6. Building a better IGFBP-2

The IGFBPs, numbered IGFBP-1–6, have molecular masses in the range of 22–31 kDa (216–289 amino acids; [10,59]). Two important structural features in this protein family are: (1) the presence of three distinct domains (denoted as N-terminal, central and C-terminal) and (2) presence of 16–18 cysteines (20 in IGFBP-4) that are distributed within the N- and C-terminal domains and that form 8–9 disufide bonds [10,59]. The cysteines are predominantly located in the N-terminal domain (10–12 in number) with the C-terminal domain containing of 6 cysteines. The overall sequence similarity between the IGFBPs ranges from 45 to 60% with conserved residues present primarily in the N- and C-terminal domains. A considerable variation exists in the central domain thus proving that this domain is not essential to IGF-binding activity.

The biological activities of the IGFBPs can be broadly classified as IGF-dependent and IGF-independent [60,61]. The former involves the modulation of IGF-1/2 activity by competition with the IGF-1R for ligand binding. IGFBPs bind strongly to the IGFs ($K_D \sim 300$–700 pM) ensuring that all circulating IGF in the blood stream is sequestered and that the access of the IGFs to IGF-1Rs is effectively attenuated when an IGFBP is present. The binding affinity of IGF-1 for the IGFBPs is greater than its affinity towards the IGF-1R [10,59–61]. However, the relative affinities of IGF-1 and IGF-2 vary for the different IGFBPs with IGFBP-1,3,4 having higher affinities for IGF-1 compared to IGF-2 and vice versa for IGFBP-2,5,6 [62,63]. Once bound, ligands are released upon proteolysis of the IGFBPs [64]. Free ligand is then available to subsequently bind to and activate the cell surface receptor. It is now understood that the binding sites for IGF-1 are located in both the N-terminal and C-terminal domains, with the central domain having sites for proteolysis and post-translational modifications [10,59–61]. The IGF-independent actions of the IGFBPs involve activities that are independent of their IGF-binding properties. A number of additional IGF-independent actions have been reported for the IGFBPs and a comprehensive discussion of these actions have been reported elsewhere (reviewed in [4,60,61]). Germane to this discussion is the presence of an RGD motif within the C-terminal domain of IGFBP-2 (and IGFBP-1) that engages of α5β1 integrins, thereby representing the most physiologic and molecularly defined IGF-independent action of IGFBP-2 [65]. The net effect of α5β1 integrin engagement by IGFBP-2 is stimulation of a signaling cascade leading to Akt activation independent of IGF-1R signaling. Accordingly, elevated IGFBP-2 levels are a negative prognostic risk factor for invasive glioma due to this signaling paradigm and its ability to enhance cell migration and invasion [40,65].

While the biological actions of the IGF1-IGFBP-IGF1R axis have been extensively studied, an understanding of the IGF-IGFBP

Table 2
Current IGFBP 3D structures and their complexes with IGF-1.

Protein	Structural domain	Complex	Experimental method	PDB code
IGFBP-1	C-domain	Uncomplexed	X-ray	1ZT3, 1ZT5
IGFBP-2	C-domain	Uncomplexed	NMR	2H7T
IGFBP-4	N-domain	Complex with IGF-1	X-ray	2DSP
				1WQJ
		Ternary complex with IGF-1 and C-domain of IGFBP-1	X-ray	2DSQ
	N- and C-domain	Ternary complex with IGF-1	X-ray	2DSR
IGFBP-5	N-domain (residues 58–111)	Complex with IGF-1	X-ray	1H59
	N-domain (residues 40–92)	Uncomplexed	NMR	1BOE
IGFBP-6	N-domain (residues 25–69)	Uncomplexed	NMR	2JM2
	C-domain	Uncomplexed	NMR	1RMJ

interactions on a structural level is incomplete. The three-dimensional (3D) structures of full-length IGFBPs have not yet been determined, though considerable structural information is available from studies carried out on individual domains from IGFBP-1-6 [66–73]. A critical challenge in the structural characterization of full-length IGFBPs has been the difficulty in expressing these proteins at levels suitable for NMR/X-ray crystallography analysis. We recently reported the first high-yield expression and structural characterization of full-length recombinant human IGFBP-2 (rhIGFBP-2) in *Escherichia coli* [74]. This opens up new avenues to carry out structure-based functional studies in this protein family. Table 2 lists the high-resolution 3D structures obtained thus far for the individual domains from different IGFBPs and their complexes with IGF-1 using NMR or X-ray crystallography. Based on their structures and disulfide-bonding pattern, the IGFBPs are known to be thyroglobulin type-1 domain homologues. Both the N-terminal and the C-terminal domains are of α/β type consisting of 30–40% of residues in regular secondary structural elements and 60–70% in unstructured regions. Fig. 2 depicts the 3D structure of the N-terminal domain of IGFBP-4 and the C-terminal domain of IGFBP-2 determined by X-ray crystallography and NMR, respectively. Also shown is a ternary complex involving the N- and C-terminal domains of IGFBP-4 and IGF-1 [72]. The central or 'linker' domain in all IGFBPs is largely unstructured and contains sites of post-translational modification and proteolysis.

Studies involving site directed mutagenesis have identified key residues in IGFBPs that are required for binding the IGFs (reviewed in [75]). These studies have also revealed that both the N- and C-terminal domains in IGFBPs are essential for IGF-1/2 binding. It has been shown that truncated IGFBP molecules lacking the N- or C-terminal domains have considerably reduced binding affinity for the IGFs compared to the intact full-length protein. One such study in our laboratories focused on the binding affinities of truncation mutants of IGFBP-2 for IGF-1 [76]. This study has provided valuable insights into IGF-binding and is briefly discussed below.

7. Analysis of IGF-1 binding by IGFBP-2 truncation mutants

All six IGFBPs contain a CWCV motif in their C-terminal domain. In a previous study involving IGFBP-2, residues both up- and downstream of this motif were identified to be involved in IGF-1 binding [77]. Hence to test the contribution of the polypeptide segment of downstream of the CWCV motif in IGFBP-2 (residues 247–250), three different truncation mutants comprising residues 1–190, 1–248 and 249–289 were cloned and expressed in *E. coli* [76]. Although high (K_D = 7 nM), IGFBP-2$_{1-248}$ exhibited a 20-fold reduction in binding affinity compared to the full-length IGFBP-2 (K_D = 0.35 nM) and IGFBP-2$_{1-190}$ had a binding affinity indistinguishable from that of IGFBP-2$_{1-248}$. An important result was borne out by kinetic studies, which revealed that dissociation of bound IGF-1 from IGFBP-2$_{1-248}$ was considerably faster than the full-length protein. This implied that the region downstream of the CWCV motif provides stability to the IGFBP-2/IGF-1 complex, accounting for the slower dissociating rates observed in full-length IGFBP-2 as compared to IGFBP-2$_{1-248}$.

8. Self-assembly of IGFBP-2 truncation mutants into nanotubular structures

To further understand the structural basis of the above observation, IGFBP-2$_{249-289}$ was subjected to structural analysis using NMR spectroscopy. While the native form of IGFBP-2$_{249-289}$ has two cysteines (C-249 and C-270), the polypeptide fragment used in our study had an additional cysteine at position 281 (R281C). Under reducing conditions such as in the presence of 1 mM β-mercaptoethanol the protein remained a monomer. However, upon removal of β-mercaptoethanol by ultrafiltration, it was found to spontaneously self-assemble into nanotubular structures several micrometers long [78]. This was investigated in detail using transmission electron microscopy, NMR and fluorescence microscopy and found to be the result of inter-molecular
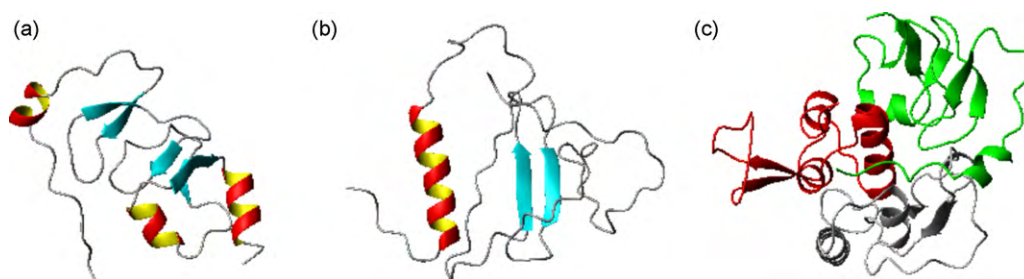

Fig. 2. *IGFBP structures obtained by NMR or X-ray crystallography.* (a) 3D structure of the N-terminal domain of IGFBP-4 (PDB code: 2DSP) and (b) the C-terminal domain of IGFBP-2 (PDB code: 2H7T). (c) Also shown is a ternary complex involving the N- and C-terminal domains of IGFBP-4 with IGF-1 (PDB code: 2DSR).

disulfide bonds formed due to the presence of an odd number of cysteines in the polypeptide fragment. This observation opens up avenues for novel biomedical applications and at the same time raises some important questions. For instance, is it possible that polypeptides resulting from proteolysis of IGFBPs also undergo such ordered aggregation if they end up with an odd number of cysteines? Could IGFs play any role in stabilizing or de-stabilizing such aggregates influencing, in turn, the efficiency of proteolysis? There are many instances where the IGFBP fragments resulting from proteolysis contain an odd number of cysteine residues [64]. The structural properties of such IGFBP fragments remain to be investigated.

A potential application of the nanotubes described above lies in targeting integrin positive tumors, taking advantage of the fact that IGFBP-2$_{249-289}$ contains an RGD motif, known to be recognized by $\alpha5\beta1$ integrin [65]. In recent years, highly efficient tumor targeting systems based on carbon nanotubes have been proposed [79,80]. In these applications, soluble carbon nanotubes were functionalized with an RGD peptide in order to target cell surface integrins. The presence of nanotubes in vivo is probed using Raman imaging [80]. The IGFBP-2$_{249-289}$ nanotubes we have described may be similarly used and their inherent tyrosine fluorescence [78] exploited for detection or monitoring. Work in this direction is currently in progress in our laboratories.

9. IGF-antagonist based cancer therapeutics

A few peptides have been developed which mimic the IGF-binding domain of IGFBPs [81]. One of these peptides blocked IGF-1 stimulated insulin receptor autophosphorylation [81]. This peptide had a structure similar to the IGF-binding domain of IGFBP-5 [66]. Further developments can benefit from structural details of how the N- and the C-terminal domains of IGFBPs together bind IGF thereby allowing the use of rational and combinatorial protein engineering approaches. A recent review on developing therapeutic proteins by engineering ligand–receptor interactions discusses these approaches [82]. The recent structural studies on the ternary complex of N- and C-terminal domains of IGFBP-4 with IGF-1 are a significant step in this direction (Fig. 2; [72]). However, two major challenges will have to be tackled for developing IGFBPs as IGF-antagonist based therapeutics: (1) the IGFBPs should be protease resistant so as to be more effective in inhibiting IGF-1R signaling and (2) IGF-independent actions should be handled so that they do not stall the beneficial effects of IGF-1 binding, including integrin engagement by IGFBP-1 and IGFBP-2 through their RGD motifs. The first objective can be achieved by developing protease resistant forms of IGFBP. A number of proteases regulate IGFBP levels extracellularly, dissociating the IGFBP-IGF complex thereby increasing IGF-1/2 available for interacting with the IGF-1R [64]. This is based on the differential effects of IGFBP-3 in tumor vs. normal prostate cells, wherein IGF-1 bioavailability is increased via PSA-mediated IGFBP proteolysis [83]. Thus, there is a need for understanding the structural mechanisms involved in proteolysis in order to develop protease resistant IGFBPs with enhanced IGF-inhibitory actions. The second objective is more difficult as a number of IGF-1 independent actions have been reported [60,61]. However, a first step in the case of IGFBP-2 is to modify its RGD motif by mutagenesis to abrogate integrin binding capacity.

10. Increasing the IGF-binding affinity of the IGFBPs

Developing IGFBPs as IGF-antagonists for cancer therapeutics also leads to the question of whether the IGFBP binding affinity for the IGFs can be further enhanced. One starting point for engineering improved antagonists is to introduce mutations with the goal of enhancing their IGF-binding affinity [82]. An alternative

approach is to develop novel chimeric protein constructs containing the N- and C-terminal domains taken from different IGFBPs. This exploits the variations in affinity of N- and C-terminal domains of different IGFBPs in binding IGFs [75]. For instance, the C-terminal domain of IGFBP-2 can be combined with the N-terminal domain of IGFBP-3 or vice versa. A chimeric protein produced this way may bind to IGF-1 more efficaciously than either individual protein. Chimeric proteins have been used in the past in drug development to impart properties from each of the parent proteins to the resulting drug [84].

11. Anti-diabetic effects of IGFBP-2

Little is known about the potential toxicities associated with the use of IGFBPs as therapeutic agents in cancer or other diseases. What is known comes from studies in mice where IGFBP-2 overexpression can promote glioma development and progression [85]. Although this may be true for other cancers [86], this effect is likely due to integrin engagement by the IGFBP-2 RGD motif. However, IGFBP-3 was found to suppress cancer, by inhibiting prostate cancer xenograft growth [87]. IGFBP-1 and IGFBP-2 impact metabolic regulation [40] with the levels of these two proteins decreasing in type 2 diabetes as a result of hyperinsulinemia [88]. Consistent with this, compared to wild type animals, IGFBP-2 transgenic mice exhibit a lean phenotype, are protected against developing age-related glucose intolerance, insulin resistance and high blood pressure and are resistant to developing obesity and insulin resistance when fed high-energy or high-fat diets [89]. In addition to being obesity-resistant, these animals have decreased leptin levels, all of which suggest that IGFBP-2 may be a factor in obesity prevention [89]. A recent microarray analysis of leptin action revealed up-regulation of IGFBP-2 in the livers of leptin vs. vehicle treated ob/ob mice [90]. This observation was further validated by acute IGFBP-2 overexpression in ob/ob, type 1 and type 2 diabetic mice, using adenoviral infection, and in all cases plasma glucose and insulin levels were reduced. This improved glycemic control, which was independent of weight loss and food intake, was also observed in leptin-resistant animals, confirming that IGFBP-2 acts downstream of leptin action [90]. These findings provide additional rationale for considering IGFBP-2 as a cancer therapeutic by underscoring the reduced diabetic symptoms IGFBP-2 therapy would likely have.

12. Conclusion and perspectives

It is clear from the increased number of drugs under development, that targeting the IGF-1R/IGF system is a viable approach to therapeutics for cancer and other diseases. The success of these approaches will be related to their limited toxicities and lack of induction of significant resistance to treatment. This raises the question of how best to monitor patients for therapeutic outcome and whether pre-selecting patients for those who would be predictably more sensitive to therapeutic intervention. Given that IRS-1 involvement is an absolute requirement for IGF-1R signaling in cancer, it has been suggested that it may serve as a biomarker. The question of utility and cost effectiveness of biomarker analyses in phase I clinical trials has recently been the subject of multiple reviews [91]. In addition to monitoring biomarkers during therapy, there are also predictive biomarkers to consider. As we progress into the era of personalized medicine, pre-selection of patient populations representing those expected to be the highest responders to a given drug regimen will be possible [92–95]. In this context, Yee and coworkers demonstrated that IRS protein expression is required for mAb down-regulation of the IGF-1R to yield an inhibitory response [96]. IRS-1 has been considered to be the biomarker of choice in cancer, with its

presence indicating a cell's sensitivity to IGF-1R targeting drugs [2]. Recently, tissue microarray analysis of patients with invasive breast cancer revealed that elevated levels of phosphorylated IGF-1R/IR were prognostic of poor survival, while total IGF-IR levels were not [97] supporting the contention that evaluating phosphorylated IGF-1R and IR might serve as a predictive biomarker for response to IGF-1R TKIs.

Finally, resistance to mAbs and RTKIs targeting the IGF-1R including compensatory activation of other growth factor RTK pathways, such as the EGFR pathway [98], have been and will continue to be present on the horizon. As newer drugs and potential combination therapies based on the identification of novel molecular targets come into existence, the toxicities of many of the current drugs may become less problematic. Additional, novel targeting opportunities exist based on crosstalk that occurs between IGF-1Rs and EGFRs [99], VEGFRs [100] and highly druggable GPCRs [101]. There is much to look forward to in the context of targeting the IGF system and developing personalized therapies to reduce the metastatic potential of many cancers.

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