Molecular targeting of growth factor receptor-bound 2 (Grb2) as an anti-cancer strategy

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Growth factor receptor-bound 2 (Grb2) is a ubiquitously expressed adapter protein that provides a critical link between cell surface growth factor receptors and the Ras signaling pathway. As such, it has been implicated in the oncogenesis of several important human malignancies. In addition to this function, research over the last decade has revealed other fundamental roles for Grb2 in cell motility and angiogenesis – processes that also contribute to tumor growth, invasiveness and metastasis. This functional profile makes Grb2 a high priority target for anti-cancer drug development. Knowledge of Grb2 protein structure, its component Src homology domains and their respective structure–function relationships has facilitated the rapid development of sophisticated drug candidates that can penetrate cells, bind Grb2 with high affinity and potently antagonize Grb2 signaling. These novel compounds offer considerable promise in our growing arsenal of rationally designed anti-cancer therapeutics. *Anti-Cancer Drugs* 17:13–20 © 2006 Lippincott Williams & Wilkins.

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Introduction

Growth factor receptor-bound 2 (Grb2) is a ubiquitously expressed adapter protein that is essential for a variety of basic cellular functions. First discovered in the nematode *Caenorhabditis elegans*, the *sem5* (sex muscle abnormal) gene is an invertebrate homolog of human *grb2*. The *sem5* product mediated signaling between a receptor protein tyrosine kinase (RTK) and a Ras protein involved in the induction of vulva formation [1]. The human *grb2* gene, also a signal transducer, was independently isolated by screening an expression library with the tyrosine-phosphorylated C-terminal tail of the epidermal growth factor receptor (EGFR) [2]. The name Grb2 reflects this method of cDNA isolation. Human *grb2* maps to the long arm of chromosome 17 (17q24–q25), which is known to be duplicated in leukemias and solid tumors. The 654-bp coding region is distributed among five exons over a total length of 18 kb. The mature 217-amino-acid Grb2 protein has homology to non-catalytic regions of c-Src: exons 1 and 2 encode an N-terminal Src homology 3 (SH3) domain, which binds proline-rich regions within other proteins, exons 3, 4 and 5 bp of exon 5 encode the central Src homology 2 (SH2) domain, which binds tyrosine-phosphorylated peptide sequences, while the remainder of the relatively large fifth exon encodes a second, C-terminal, SH3 domain [2,3]. The *grb2* gene is highly conserved among species: Sem-5 shares 58% amino acid identity with human Grb2, *Drosophila* Grb2 is 66% identical, and both rat and mouse homologs are over 99% identical. Grb2 expression has been shown to be critical for normal development. Grb2-null mouse embryonic stem cells are unable to differentiate into parietal and visceral endoderm, leading to early developmental arrest and embryonic lethality [1,4–7]. Mice with haploinsufficiency of the *grb2* gene survive embryonic development, but display defective T cell signaling [8,9].

Grb2 signaling in normal cellular functions and in cancer

Like many other intracellular effectors of mitogenesis, Grb2 binds to tyrosine phosphorylated proteins via its SH2 domain, linking Grb2 function temporally and spatially with the regulation of tyrosine phosphorylation in these pathways. SH2 domains directly recognize phosphotyrosyl residues (pY) within the context of peptide or protein ligands [10], with additional secondary binding interactions within 2–3 amino acids C-terminal to the pY residue that introduce differential affinity toward SH2 domain subfamilies [11,12]. SH2 domain-mediated recognition by Grb2 is specific for the phosphopeptide motif pYXNX, where N is asparagine and X is any residue. Growth factor RTKs, including those for epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (TrkA/B), platelet-derived growth factor (PDGF), colony-stimulating factor-1 and hepatocyte growth factor (HGF), as well as non-RTKs

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such as Bcr–Abl and focal adhesion kinase (FAK), intracellular effectors such as insulin receptor substrate-1 and Shc, and phosphotyrosine phosphatases such as SHP-2 (PTPN11) and receptor-like tyrosine phosphatase-α [13], all possess the pYXNX motif recognized by the Grb2 SH2 domain. Note that the environmental cue leading to protein tyrosine phosphorylation on an appropriate Grb2 recognition motif is independent of Grb2 interaction; thus, ligand-independent EGF receptor (EGFR) activation, such as growth hormone-induced EGFR tyrosine phosphorylation by JAK2, also leads to Grb2-mediated mitogen-activated protein kinase (MAPK) pathway activation and c-*fos* expression [14]. Similarly, mechanical stress leading to increased angiotensin II production and transactivation of EGFR and other intracellular kinases implicates Grb2 recruitment in cardiac hypertrophy and myocardial remodeling [9].

In many mitogenic signaling pathways, recruitment of Grb2 from the cytosol, where it is already bound to the guanine nucleotide exchange factor SOS1 via its N-terminal SH3 domain, brings SOS1 in close proximity to Ras at the plasma membrane. Ras, a small GTPase in the GDP-bound inactive state in quiescent cells, then undergoes nucleotide exchange of GDP for GTP, which facilitates binding of the serine/threonine protein kinase Raf1 and its subsequent activation. This initiates a cascade of kinase activation: activated Raf1 phosphorylates and activates MEK1/2, which in turn phosphorylate and stimulate the MAPKs ERK1/2. Activated ERKs translocate to the nucleus and phosphorylate transcription factors such as Elk-1 and Myc, activating gene expression. In parallel, the phosphatidylinositol-3-kinase (PI3K)/Akt pathway is activated via Gab1, which is bound to the Grb2 C-terminal SH3 domain in many epithelial cell types [15]. The gene expression programs activated by these pathways initiate a spectrum of fundamental cellular activities including proliferation, differentiation and survival, and both pathways are critically involved in a wide variety of human cancers. The SH3 domains of Grb2 also bind Cbl, an E3 ubiquitin ligase that functions as an early attenuator of RTK signaling via receptor ubiquitination as well as a facilitator of receptor endocytosis. RTK endocytosis into clathrin-coated pits and vesicles is also facilitated by dynamin 1, another Grb2 SH3 domain-interacting protein that functions as a GTPase essential for coated vesicle formation. In T cells, activation of the T cell antigen receptor (TCR) results in tyrosine phosphorylation of the membrane-associated adaptor protein LAT and association of its cytosolic domain with the Grb2 SH2 domain. In these cells the SH3 domains of Grb2 also bind Vav family proteins – guanine nucleotide exchange factors for Rho family GTPases. These interactions are essential for TCR-induced calcium flux and activation of the MAPK cascade, ultimately leading to T cell proliferation and effector functions [16,17].

In addition to its role as a receptor-proximal adaptor protein, Grb2 participates directly in the regulation of actin filament formation and actin-based cell motility. Grb2 is a critical link between Wiskott–Aldrich syndrome protein (WASp) and the actin cytoskeleton; WAS patients show defects in T cell polarization and migration in response to physiologic stimuli, resulting in thrombocytopenia, eczema and immunodeficiency [18]. Studies of WASp function and the intracellular motility of invasive microbial pathogens such as *Listeria monocytogenes* and vaccinia virus also helped to elucidate the important role for Grb2 in directly promoting actin-based motility [19,20]. These microbes invade eukaryotic cells, and use a limited number of microbial surface proteins to harness host cell actin and associated regulatory molecules for propulsion through the host cytoplasm. Unraveling these pathogenic events helped to define the roles of several eukaryotic proteins in normal actin-based motility. In most mammalian cells, the WASp family member N-WASp interacts with the Arp2/3 complex and G-actin to stimulate actin polymerization. N-WASp activity is enhanced by other effectors such as Nck, Cdc42 and Grb2; disruption of Grb2 SH3 or SH2 domains diminishes actin polymerization, and thus actin-based motility, suggestive of a critical role for Grb2 in motility independent of its role in mediating growth factor stimulated cell motility [18,20].

Several of the functions served by Grb2 outlined above make it an attractive target for anti-cancer therapeutic development. In addition, many of the signaling pathways in which Grb2 functions are critical for vasculogenesis, angiogenesis and lymphoangiogenesis, which further strengthen its candidacy as an anti-cancer drug target. Vascular endothelial growth factor (VEGF) and basic FGF (bFGF) are among the most potent regulators of angiogenesis, and share intracellular signaling mediators with a variety of angiogenesis signaling pathways [21]. VEGF-A-induced phosphorylation of KDR/VEGF receptor (VEGFR) 2 induces the recruitment of Shc, Grb2, Nck and formation the Shc–Grb2 complex. The FLT4L kinase, an alternatively spliced form of the VEGFR3/FLT4 receptor, binds Grb2 directly through its cytoplasmic region and recruits a second pool of Grb2 by phosphorylating tyrosine residues within appropriate sequence motifs in Shc [22]. As in non-endothelial cell types, Grb2 acting at this level is believed to link receptor activation to cell cycle progression and cell motility via the Sos/Ras and Rac1/Rho pathways, respectively [22–24]. The known role of HGF in angiogenesis [25] has prompted investigations into the ability of Grb2 SH2 domain-binding antagonists to block HGF-stimulated Grb2 signaling at this level, with primary effects on cell motility and motility-dependent processes [26,27].

Grb2 also acts as a critical downstream intermediary in several other angiogenic signaling pathways, including

PDGF, ELK and Eph, suggesting that these pathways may also be susceptible to blockade by Grb2 SH2 domain binding antagonists. PDGF is implicated in different biological processes such as vascular remodeling, wound healing and cancer [28,29]. Consistent with the spectrum of pathways disrupted by Grb2 antagonism, it has also been observed that the Grb2-binding antagonist C90 inhibits PDGF-BB-induced endothelial cell migration [27]. In endothelial cells, membrane-bound LERK-2 stimulates ELK tyrosine phosphorylation and recruitment of Grb2 and Grb10 via their SH2 domains, signaling the assembly of microvascular endothelial cells into capillary-like cords [23]. Interestingly, natively expressed Eph-related receptors, including ELK, Mek4 and Eck, do not signal proliferative responses upon ligand binding [23], reinforcing the importance of Grb2 in mediating motility and morphogenic responses.

As in vasculogenesis and angiogenesis, the critical role of Grb2 in signaling epithelial morphogenesis during development and tissue regeneration and repair make it an excellent target for strategies aimed at blocking pathological morphogenic processes such as the spread of solid tumors through local invasion and metastasis. The HGF/c-Met signaling pathway, essential for normal vertebrate development, in particular has been implicated in the progression and systemic spread of colon, breast, lung, thyroid and renal carcinomas, melanoma, and several sarcomas, as well as glioblastoma [15,30]. The inappropriate expression of c-Met in certain mesenchymal cells can lead to a carcinogenic transformation in which the tumor cells express both mesenchymal and epithelial markers [15]. Inherited mutations in c-Met that result in constitutive activation of the HGF signaling pathway are associated with human renal papillary carcinoma [31,32]. Importantly, in addition to its mitogenic activity, the ability of HGF to initiate a program of cell dissociation and increased cell motility coupled with increased protease production has been shown to promote cellular invasion through extracellular matrix substrates and is correlated with tumor metastasis *in vivo* [15,33,34]. The importance of Grb2 in the HGF/c-Met signaling pathway is also well established, largely via studies in which site-directed mutagenesis has been used to alter the C-terminal multifunctional docking site containing the Grb2-binding motif in c-Met and/or the oncogenic Tpr-Met protein.

Using a Tpr-Met mutant that was completely unable to associate with Grb2 protein, Fixman *et al.* showed that transformation of cultured cells was dependent on signaling downstream of Grb2 and Shc, while the activation of PI3K was not sufficient for transformation [35]. Duplication of the Grb2-binding site increased the transformation ability of Tpr-Met as observed by colony formation in soft agar, although scatter activity, thought to be instrumental in metastasis, remained unchanged [36]. Indeed, this mutant was impaired in invading extracellular matrices and forming metastatic tumors in nude mice [37], suggesting that conversion of the second SH2 recognition motif in the multifunctional docking site to the Grb2-preferred sequence (pYXNX) may have interfered with the binding of another intracellular effector that was critical for invasion and metastasis. Consistent with this result, optimization of the multifunctional docking site to bind specific effectors demonstrated that activation of both PI3K and Grb2 pathways was required to elicit an invasive response and metastatic phenotype in cultured cells [38]. A later study utilizing engineered Tpr-Met docking site mutants again confirmed the importance of Grb2 in cell transformation and experimental metastasis, although clearly more work is needed to completely define the roles of Grb2, Shc, Gab1 and PI3K signaling in normal and oncogenic signaling [39].

Grb2 signaling has also been implicated in the pathogenesis of several specific human malignancies. Among the most intensively studied malignancies, chronic myelogenous leukemia (CML) is frequently characterized by the presence of a reciprocal translocation between chromosome 9 and 22, giving rise to a chimeric Bcr–Abl gene (Philadelphia chromosome). In patients with Philadelphia chromosome-positive CML, Y177 in the BCR region of the Bcr–Abl tyrosine kinase oncoprotein is able to bind the Grb2 SH2 domain, linking the chimeric protein to activation of the Ras pathway [40]. Furthermore, Grb2 mutants with a deletion in one of the SH3 domains impair the transforming ability induced by Bcr–Abl [41], highlighting the importance of SH3 domain interactors, such as SOS1, in the process. These findings suggest that Grb2-binding antagonists may someday provide an effective alternative or adjunct therapeutic strategy for CML patients resistant to STI-571 (Gleevec), an antagonist of ATP binding by the Abl tyrosine kinase.

Grb2-mediated ErbB2/Neu signaling is thought to play a critical role in breast cancer onset, progression and metastasis. Janes *et al.* demonstrated that in human breast cancer cells, overexpressed erbB-2 was associated with the Grb2–SOS1 complex, activating the Raf/MEK/MAPK pathway through Ras [42]. Grb2 itself was found to be overexpressed in three breast cancer cell lines [7]. Interestingly, overexpression of Grb2 has been reported in human breast cancer in conjunction with overexpression of SHP-1 [43] and this combined upregulation enhanced downstream signaling through the MEK/MAPK pathway. Cheng *et al.* [7] stressed the importance of *grb2* gene dosage in the onset and development of mammary carcinomas as a possible signal amplification mechanism downstream of active tyrosine kinase receptors. These Grb2-mediated effects would be expected to contribute to a loss of cell cycle control and enhanced proliferation, as well as increased cell motility and invasion, as shown

recently in the context of keratinocyte growth factor (KGF) signaling [44]. That study demonstrated growth factor-stimulated upregulation of Grb2 gene expression and inhibition of KGF-stimulated motility via down-regulation of Grb2 protein or via MEK inhibition, illustrating the criticality of Grb2 in the overall KGF response of breast cancer cells. In addition to breast cancer, Grb2 and SOS1 overexpression was observed in four human bladder cancer cell lines in the absence of EGFR overexpression or H-Ras mutation [45], and activation of the α_2-macroglobulin receptor in the highly metastatic 1-LN prostate cancer cell line induced significant Grb2, SOS1, Shc and Raf-1 expression, and potentiated cell proliferation [46].

In summary, since its discovery 13 years ago the pivotal role of Grb2 in oncogenic signaling, among other important functions, has been firmly established. As a receptor proximal effector in a variety of cytokine and growth factor pathways, Grb2 is required and in many instances sufficient for activating the Raf/MEK/MAPK cascade leading to cell cycle progression. Additional critical roles in promoting cell motility, invasion, metastasis and angiogenesis, coupled with ubiquitous expression and evidence of overexpression in specific cancers, make Grb2 a high priority target for anti-cancer drug development.

Anti-cancer drug development strategies targeting Grb2

A wide variety of experimental strategies have been used to block signaling by Grb2, furthering our understanding of Grb2 function at the cellular, tissue and organismal levels. With continued development many of these strategies, such as anti-sense suppression of Grb2 expression, may become viable pharmaceutical approaches. However, widespread interest in the molecular basis of signaling via SH3 and SH2 domains in general has also fostered a detailed analysis of Grb2-associated proteins and the functional consequences of specific binding events, as well as extensive structural studies of Grb2–ligand interactions. This information, in turn, has facilitated the rapid development of peptide, peptidomimetic and synthetic small-molecule antagonists of these interactions with pharmaceutically desirable potency and selectivity profiles. Grb2 SH3 and SH2 domains have been successfully targeted for signaling blockade at the level of proof-of-concept; at present Grb2 SH2 domain antagonists have undergone more extensive development as anti-cancer drug candidates.

A variety of recognition motifs for SH3 domains have been discovered; peptides associated with SH3 domains share a common left-handed polyproline type II helix and a minimal consensus sequence PXXP [47]. Basic residues also contribute to the orientation of SH3 ligands: N-terminal basic residues confer binding to the SH3 domain with an N–C orientation, whereas basic residues at the C-terminus of the peptide ligand confer the reverse orientation [47]. The Grb2 SH3 domain appears to prefer the latter class of protein ligands. While Grb2 is expressed in a wide spectrum of cellular contexts, its SH2 domain binds primarily to activated growth factor receptors directly or indirectly through a 'linker' protein such as Shc or SHP-2, resulting in a predominant functional role in mediating mitogenic and motogenic signals. In contrast, the Grb2 SH3 domain binds to proteins with more diverse functions, some of which mediate mito- or motogenic signals (such as SOS1, MEKK1 and N-WASp) and others that attenuate receptor signaling or target receptors for degradation (such as Cbl, PEST and Dab-2) [48]. Thus, at the outset, targeting the Grb2 SH3 domain presents both structural and functional complexities that must be addressed in the development of drugs with selective and potent anti-cancer activity. Efforts to target the Grb2 SH3 domain have focused on blocking the interactions with SOS1 [49,50]. To attain simultaneous interactions with both SH3 domains of Grb2, peptidimers were designed by coupling two proline-rich sequences from SOS1 using linkers of different sizes. Some of these peptides have shown very high affinities for Grb2, with K_d values in the nanomolar range, and the ability to efficiently antagonize Grb2–SOS1 interaction *in vitro* [49,50].

SH2 domains are highly conserved amino acid sequences composed of approximately 100 residues and are the predominant recognition motif for phosphotyrosine found in proteins [51]. By binding to pY-containing proteins, SH2 domains provide the proximity needed for various protein–protein interactions performed by other domains in the interacting proteins; thus SH2 domains are observed to participate in a broad spectrum of biochemical processes. Tight temporal and spatial regulation of protein tyrosine phosphorylation provides fundamental control over these widely distributed binding elements: in the absence of a phosphoryl group, most SH2 domains bind poorly to their respective recognition motifs in target proteins. Early comparative analysis of SH2 domains using random phosphopeptide libraries revealed another intrinsic level of regulation – neighboring amino acid residues on the carboxyl side of the pY residue dictate preferences among specific SH2 domains. The Src SH2 domain, for example, selectively bound the peptide pYEEI, while the Grb2 SH2 domain displayed a striking preference for pYI/VNX (X is any residue) where the asparagine residue at position pY + 2 was invariable [12]. Later structural studies provided the physical basis for these two basic types of observed SH2 domain selectivity: the Src SH2 domain accommodated the pY and I residues in two deep pockets with the intervening residues binding in an extended chain between them, while the Grb2 SH2 domain was deeper and more

compact, forcing the cognate phosphopeptide into a β-turn conformation that was anchored at the pY and the pY + 2 positions [52].

The development of SH2 domain-binding antagonists has followed several strategic paths [53]. In advancing from simple phosphopeptides, phosphonate- and carboxylate-based pY mimetics with resistance to intracellular phosphatases were substituted for pY [54,55]; these substitutions have undergone continuous refinement [56,57]. Modifications to the structure of peptidic antagonists to circumvent poor cell permeability due to the presence of negatively charged groups progressed in parallel. Further development of Grb2 SH2 domain antagonists focused on the rational design of peptidomimetics and non-peptidic compounds that exploited the unique structural features of this domain. These efforts included optimization of the N- and C-terminal groups and of the modified hydrophobic residue X of the minimum pY–X–N peptide, the design of non-phosphorylated cyclic peptides and the search for peptidomimetics retaining little or no peptidic character [55,58–61]. Combined with these improvements, replacement of the I/V residue at position + 1 with 1-aminocyclohexane carboxylic acid to further stabilize the β-turn conformation produced a selective and high-affinity (below 50 nmol/l) Grb2 SH2 domain antagonist [62]. Many of these compounds have exhibited high binding affinity in extracellular assays, although in physiological contexts where Grb2 proteins reside in intracellular compartments persistent concerns regarding the ability of highly anionic phosphate-mimicking groups and the extended peptidic structures of many inhibitors to transit cell membranes have driven further refinement of peptidomimetic and non-peptide antagonists. Covalent conjugation of Grb2 SH2 domain-binding peptides with carrier peptides, derived from secreted protein signal sequences or from the third helix of the Antennapedia protein, resulted in the rapid shuttling of inhibitors across lipid bilayers [63,64]. Protection of anionic charge groups through pro-drug derivatization has also been used to improve the potencies of Grb2 SH2 domain-directed peptides in intact cells [49,50,65].

While antagonist-binding selectivity among available SH2 domains inside the cell is not yet firmly established, there is substantial evidence that Grb2–RTK interactions, and several functional consequences thereof, have been potently blocked. The synthetic Grb2 SH2-binding antagonist CGP78850 and related pro-drugs potently blocked EGFR–Grb2 and Shc–Grb2 interactions, inhibiting serum-stimulated mitogenesis and anchorage-independent growth in intact MDA-MB-468 human breast cancer cells, and causing phenotypic reversion of *neu* transformed fibroblasts [65]. These compounds also induced expression of the cell cycle inhibitors $p21^{Waf1/Cip1/CAP1}$ and $p27^{Kip1}$, and failed to block cellular transformation by oncogenic *raf* or mutated *ras*, consistent with selective antagonism of Grb2 SH2 domain-mediated RTK signaling. Similar compounds also inhibited HGF-stimulated motility in A431 and Madin–Darby canine kidney cells, as well as motility-related events such as EGF-induced membrane ruffling and Rac translocation, providing early evidence that Grb2 signaling may be required for motogenic as well as mitogenic growth factor responses [66].

Small synthetic Grb2 SH2 domain-binding antagonists with novel phosphomimetic groups and lacking pro-drug derivatization were reported by Yao *et al.* to inhibit Grb2–$p185^{erbB-2}$ interaction in intact MDA-MB-453 human breast cancer cells, in which the *erb*B-2 gene is amplified and the $p185^{erbB-2}$ protein is overexpressed [67]. When the compounds were introduced directly into MDA-MB-453 cell lysates, a clear dose-dependent reduction in the Grb2-associated $p185^{erbB-2}$ was observed, consistent with surface plasmon resonance spectrometry (Biacore) results obtained with purified proteins [67]. Atabey *et al.* analyzed the effects of compounds similar to those reported by Yao *et al.* on HGF signaling in several intact normal and tumor cell lines, and demonstrated potent ($ED_{50} < 30$ nmol/l) blockade of c-Met/Grb2 association, cell motility, matrix invasion and epithelial tubulogenesis [26]. Unlike signaling by EGFR-related RTKs, HGF-stimulated c-Met activation results in activation of the MEK/MAPK cascade through Grb2/SOS/Ras and in parallel through Gab1-mediated PI3K activation. Thus failure of the compounds to block HGF-induced PI3K and MAPK activation, and hence mitogenesis, despite inhibition of c-Met Grb2 association, was consistent with selective pathway blockade at the level of the Grb2 SH2 domain [26]. Building on these results, Soriano *et al.* demonstrated that the same SH2 domain-binding antagonists blocked the basic morphogenetic events required for angiogenesis, including HGF-, VEGF- and bFGF-stimulated migration and matrix invasion by large vessel and microvessel vascular endothelial cells. Inhibition of phorbol ester (phorbol myristate acetate)-induced microvascular endothelial cell migration was also observed [27]. PMA is an artificial activator of protein kinase C (PKC); PKC-mediated migration plays an important role not only during angiogenesis but also during tumor development, tumor invasion and metastasis. Signaling through the VEGF receptors Flt-1 and Flk-1 is thought to occur via activation of the PI3K, PKC and MAPK pathways, ultimately leading to capillary formation *in vitro*, and to vasculogenesis and angiogenesis *in vivo* [68]. Inhibition of VEGF-driven angiogenesis *in vitro* was manifested as suppression of cord formation by microvascular endothelial cells grown in Matrigel and suppression of angiogenesis *in vivo* was also observed using the chick chorioallantoic membrane (CAM) assay with submicromolar potency [27]. These results further implicate Grb2 as an important mediator of key pro-angiogenic

events, and suggest that Grb2 SH2 domain antagonists have the potential to act as anti-cancer drugs that target both tumor and vascular cell compartments.

Summary and future directions

Considerable effort continues to be devoted to the refinement of peptidomimetic and non-peptide Grb2 SH2 domain-binding antagonists with the goals of increasing biological potency, stability and bioavailability as well as reducing cost of synthesis [69]. Direct analysis of binding association and dissociation rates via Biacore techniques has facilitated rapid improvements in drug potency through relatively subtle compositional and structural modifications. For example, global conformational constraint of an α-methyl-*p*-phosphonophenylalanine-containing peptide by β-macrocyclization resulted in pronounced elevation of binding affinity, which was achieved primarily through a decrease in the binding 'off' rate [70]. Combining these conformational constraints with improved pY mimetics including phosphonic acid and malonyl-containing diacidic phosphoryl moieties has produced Grb2 SH2-binding antagonists with single-digit nanomolar affinities [71].

Despite the broad-based effort devoted to developing Grb2 SH2 domain-binding antagonists, important questions related to ligand selectivity and intracellular localization remain unanswered. To address these issues, Shi *et al.* used two parallel strategies to label small synthetic antagonists while maintaining potent biological activity and overall antagonist structure – incorporation of a fluorophore and, independently, incorporation of biotin [72,73]. Fluorescence labeling has become a general technique for studying the intracellular accumulation and localization of exogenously administered materials. Shi *et al.* developed a high affinity Grb2 SH2 domain-binding antagonist that utilizes the environmentally sensitive nitrobenzoxadiazole fluorophore as a naphthyl replacement. This novel agent should serve as a useful tool to visualize the actions of this class of Grb2 SH2 domain-binding antagonists in whole cell systems [72]. A novel biotinylated macrocyclic SH2 domain antagonists bearing biotin functionality at the C-terminal rather than the traditional N-terminal position was also designed and synthesized. With a Grb2 SH2 domain-binding $K_{(eq)}$ value of 3.4 nmol/l, the biotin-labeled compound is among the most potent biotinylated SH2 domain-binding ligands yet disclosed [73]. This should be a useful tool for elucidating physiological targets of certain Grb2 SH2 domain-binding antagonists via streptavidin capture of drug-treated cells and immunological and/or mass spectrometric analysis of associated proteins.

Assessment of the antagonists described by Atabey *et al.* [26] and Soriano *et al.* [27] in mouse xenograft models is currently underway. No toxicity was reported for Grb2 SH2 domain antagonists in intact cells or in chick embryos treated for 72 h and displaying local inhibition of angiogenesis in the CAM [27]. Mice treated for 28 days by daily i.p. injection or continuous infusion via implanted osmotic minipumps at doses designed to exceed the ED_{50} in cultured cells by 100-fold showed no signs of toxicity or histological abnormalities in major organs (Bottaro *et al.*, unpublished observations). Selection of tumor cell lines for these xenograft studies has been based on cultured cell assays for motility and invasion stimulated by HGF. As inhibition of proliferation was not observed in those cell lines, models of metastasis (e.g. tail vein injection of tumor cells to colonize the lungs) are favored over models of primary tumor growth. In cell models where signaling pathways, such as that of EGF, display a strict dependence on Grb2 for activation of the Ras/MEK/MAPK cascade, s.c. tumor growth in mice may be the most expedient xenograft method. Given the broad spectrum of cancer types showing involvement of the Grb2/SOS1/Ras signaling pathway, a variety of animal models are well worth considering for the preclinical evaluation of these antagonists.

As a ubiquitous adapter protein that links growth factor receptors with Ras signaling, Grb2 is an attractive target for anti-cancer drug development. Additional roles for Grb2 in cell motility and angiogenesis, processes that also contribute to tumor growth, invasiveness and metastasis, further underscore its importance in cancer and broaden our strategies for drug evaluation *in vitro* and *in vivo*. Our knowledge of SH domain structure and function has facilitated the rapid design and refinement of sophisticated synthetic low-molecular-mass compounds that bind Grb2 with high affinity and demonstrate potent inhibition of Grb2 function. These compounds serve as research tools to further explore the role of Grb2 signaling in health and disease, and represent viable anti-cancer drug candidates. Current assessment in animal models for pharmacodynamics, toxicity and biological activity should contribute to optimal delivery and dosing strategies, and may help prioritize target indications for future clinical studies.

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