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Inducing Oncoprotein Degradation to Improve Targeted Cancer Therapy[1]

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Abstract

Over the past decade, inhibition of the kinase activities of oncogenic proteins using small molecules and antibodies has been a mainstay of our anticancer drug development effort, resulting in several Food and Drug Administration–approved cancer therapies. The clinical effectiveness of kinase-targeted agents has been inconsistent, mostly because of the development of resistance. The expression and function of oncoproteins and tumor suppressors are regulated by numerous posttranslational protein modifications including phosphorylation, ubiquitination, and acetylation; hence, targeting specific posttranslational protein modifications provides for an attractive strategy for anticancer drug development. The present review discusses the hypothesis that targeted degradation of an oncoprotein may overcome many of the shortcomings seen with kinase inhibitors and that the approach would enable targeted inhibition of oncogenic proteins previously thought to be undruggable.

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Introduction

The molecular complexity of cancer is reflected by the ever-increasing list of genetic drivers of oncogenesis. In preclinical models, the targeted inhibition of oncogenic pathways has been an effective strategy in many types of cancer; however, the clinical success of these drugs has been limited to a handful of targets and diseases. Because aberrant kinase activity is linked to oncogenesis, adenosine triphosphate–competitive inhibitors such as erlotinib represent the mainstay of our current drug development pipeline. Unfortunately, many oncogenic targets are thought to be undruggable using these traditional drug design strategies, and the development of drug resistance to existing targeted agents is a significant problem. Novel strategies to target oncogenic drivers are greatly needed.

X-ray crystallography, nuclear magnetic resonance spectroscopy, and molecular modeling have elucidated the three-dimensional structures of many protein targets. Insight into the structural basis of kinase function as well as the structural requirements for protein-ligand and protein-protein interactions has opened the door for novel therapeutic strategies. The identification of specific protein domains involved in chaperone binding, ubiquitination, and dimer formation allows for the development of novel agents that target oncoprotein stability and induce degradation. These strategies have the potential to overcome resistance seen with traditional kinase inhibitors.

In this article, we will review previous attempts at targeting various protein posttranslational modifications including phosphorylation. We then present an argument that the targeted degradation of an oncoprotein has several advantages over the mere inhibition of kinase activity as this strategy has the potential to affect the cellular processes of a protein that are not related to kinase activity. Furthermore, we describe how undruggable proteins such as KRAS may be targeted with this approach.

Background: Targeting Posttranslational Modifications for Cancer Therapy

Although several posttranslational modifications affect the function of an oncoprotein, almost all drug development efforts have focused on the altering the attachment of phosphate groups by protein kinases. This protein modification controls many important aspects of protein activity, localization, and stability. Many very successful

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drugs, particularly tyrosine kinase inhibitors, have been developed using this strategy (Table 1). For example, imatinib (Gleevec, Novartis) revolutionized the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors [1]. Also, epidermal growth factor receptor (EGFR) inhibitors including erlotinib (Tarceva, Genentech) have become standard of care for subsets of patients with non–small cell lung cancer and colorectal cancer [2]. Serine/threonine kinase inhibitors and multikinase inhibitors have also been developed. Vemurafenib (Zelboraf, Daiichi Sankyo) is a serine/threonine kinase inhibitor that is Food and Drug Administration (FDA) approved for patients with metastatic $BRAF^{V600E}$ mutant melanoma [3]. Sorafenib (Nexavar, Bayer) inhibits the serine/threonine kinase RAF and the tyrosine kinases platelet-derived growth factor receptor and vascular endothelial growth factor receptor. It has FDA approval for the treatment of advanced-stage renal cell carcinoma and hepatocellular carcinoma [4].

The concern with selective targeted agents is that parallel signaling pathways can compensate for the inhibition of a single kinase and resistant mutations can quickly develop. Conversely, with multitargeted kinase inhibitors, there are issues with off-target effects leading to toxicity. Also, because multiple pathways are affected, there is a poor understanding of the true mechanism of these agents in a particular tumor, making it difficult to develop further improvements in specificity or activity.

Besides phosphorylation, other posttranslational protein modifications have been identified as targets for cancer therapy. One of the earliest attempts at this strategy was inhibition of RAS isoprenylation by farnesyltransferase inhibitors [5]. More than 30 years ago, the attachment of farnesyl side chains to RAS proteins (including H-, N-, and K-RAS) was found to be critical for wild-type and mutant oncogenic protein localization and function. Farnesyltransferase inhibitors showed promise in preclinical tumor models; however, they have failed in the clinic because of the presence of alternate isoprenylation pathways through the geranyl side chain attachment. Given the presence of multiple isoprenylation modifications, dual inhibitors targeting both farnesylation and geranylation were tested; however, this strategy showed increased toxicity as approximately 30 mammalian proteins are known to undergo similar posttranslational modifications. A recent review [6] describes the many unsuccessful attempts at targeting RAS. Given its important role in several types of cancer, novel strategies are needed to target KRAS mutant-driven tumors.

Similar to phosphorylation, protein acetylation has been extensively explored as a strategy to target cancer. Histone deacetylase (HDAC) inhibitors such as vorinostat (Zolinza) and romidepsin (Istodax) are FDA approved for the treatment of cutaneous T-cell lymphoma [7]. Recently, LBH589 (Panobinostat, Novartis) in combination with dexamethasone and bortezomib was shown to slow progression of multiple myeloma in a phase III trial. The mechanism behind HDAC inhibitor cytotoxicity is complex and poorly understood. One proposed mechanism is that HDAC inhibition leads to increased expression of tumor suppressors, although several other cellular processes are altered as well.

Chaperone proteins represent a unique class of therapeutic targets. After translation, the chaperone machinery plays an important role in protein homeostasis by facilitating nascent protein maturation and folding. HSP90 is critical for the maintenance of more than 600 proteins including several oncoproteins. Even though highly potent HSP90 inhibitors such as geldanamycin and its derivatives (17-AAG or 17-DMAG) (reviewed in [8]) have been extensively studied, no HSP90 inhibitors are FDA approved because of their toxicity. Next-generation HSP90 inhibitors, such as ganetespib [9], have shown promise for improved tumor selectivity in preclinical models. Preliminary results from an ongoing clinical trial suggest that the single agent ganetespib has activity in a subset of advanced-stage non–small cell lung carcinoma (NSCLC) patients [10]. Furthermore, a phase IIB/III study evaluating the safety and therapeutic activity of ganetespib in combination with docetaxel in NSCLC found that this combination improved survival [11]. It is unclear why ganetespib is less toxic than first-generation HSP90 inhibitors. It may be due to a more tolerable dosing schedule or relatively selective degradation of oncoproteins. Future studies will improve our understanding of how to effectively use HSP90 inhibitors in patients.

Inducing Protein Degradation as an Attractive Alternative to Inhibition

Resistance to kinase inhibitors is a major challenge limiting the clinical effectiveness of these drugs. Single amino acid substitutions can make a kinase inhibitor ineffective. Given that a tumor typically contains a billion cells per cubic centimeter and the fact that tumors cells are genetically unstable, it is likely that a resistance mutation will be present. Prolonged exposure to a drug will select for the resistant population. This phenomenon has been clearly demonstrated in non–small cell lung cancer expressing EGFR with a threonine-790-methionine (T790M) point mutation [12]. Careful analysis has indicated that the T790M mutation is a *de novo* mutation, inferring that TKI treatment selects for these cells. A drug that selectively degrades a target should theoretically be effective in kinase inhibitor–resistant and –sensitive cells.

One approach to eliminating a target rather than simply inhibiting it is to deliver small interfering RNA (siRNA). Indeed, several studies suggest that the reduction of a protein by an siRNA can be effective for treating cancer. Reducing the amount of a specific protein has certain advantages over inhibition of its activity as a protein's physical presence can serve critical functions beyond its catalytic activity. One of the first examples of this concept came from the yeast (*Saccharomyces cerevisiae*) protein Pbs2p. Both the kinase activity of Pbs2p and its function as scaffolding were found to be equally important in maintaining cellular osmolarity [13]. A recent review article by Rauch et al. [14] identifies more than 70 kinases whose catalytic activity and physical presence (mainly as scaffolding proteins) are critical in disease pathogenesis. Among these kinases, some of the most relevant to cancer are in the ErbB family which includes EGFR and Her2. ERBB3 is a very interesting member of this family as it has no kinase activity but can facilitate signal transduction by forming a heterodimer with another family member [15]. Furthermore, a kinase-defective mutant of EGFR (K721M) can activate downstream signaling with assistance of ErbB2 (Her2) after EGF stimulation [16]. These findings demonstrate that ErbB family members and other receptor tyrosine kinases have important cellular functions that are independent of kinase activity.

In addition to its scaffolding role, EGFR has many other functions beyond kinase activity. EGFR was recently found to protect cells from autophagy by a kinase-independent mechanism [17]. Also, the EGFRvIII mutant can sequester the proapoptotic protein PUMA in a kinase independent manner, promoting drug resistance [18]. Furthermore, in genetically engineered mouse models, EGFR knockout is embryonically lethal [19], whereas transgenic mice

Table 1. List of Posttranslational Protein Modifications and FDA-Approved Agents as Anticancer Agents

Agent	Target	Disease Site	Clinical Results	Year FDA Approval
Tyrosine kinase inhibitors				
Trastuzumab	HER2	Metastatic HER2+ gastric cancer	OS improved 11.7 to 13.1 mo	2010
		Adjuvant therapy for HER2+, LN+ breast cancer	DFS HR 0.48	2006
		Metastatic HER2+ breast cancer	TTP improved 4.6 to 7.6 mo	1998
Imatinib	BCR-ABL c-Kit	Adjuvant therapy for GIST	Improved OS with 36 mo vs 12 mo HR 0.45	2008/2012
		Unresectable or metastatic GIST	ORR 38%	2002
		CML	Hematologic response 88% (chronic phase)	2001
Gefitinib	EGFR	NSCLC	ORR 10.6%	2003
			Two failed clinical trials	Revoked 2005
Bevacizumab	VEGFR	Metastatic cervical cancer	OS improved 12.9 to 16.8 mo	2014
		Platinum-resistant ovarian cancer	PFS improves 3.4 to 6.8 mo	2014
		Renal cell carcinoma	PFS improved 5.4 to 10.2 mo	2009
		Refractory high-grade glioma	No phase III data with non–bevacizumab-containing arm	2009
		Metastatic renal cell carcinoma	PFS improved 5.4 to 10.2 mo	2009
		Metastatic breast cancer	PFS improved 5.8 to 11.3 mo	2008
			No survival benefit	Revoked 2011
		Nonsquamous NSCLC	OS improved 10.3 to 12.3 mo	2006
		Second-line metastatic colorectal cancer	OS improved 10.8 to 13.0 mo	2006
		First-line metastatic colorectal cancer	ORR improved 35% to 45%	2004
Cetuximab	EGFR	K-ras wild type, EGFR-expressing metastatic colorectal cancer	OS improved 19.5 to 23.5 mo in K-ras wild-type tumors	2012
		Metastatic head and neck cancer	Improved OS 18.2 to 19.1 mo	2012
		Head and neck cancer with radiation therapy	OS improved 29.3 to 49.0 mo	2006
		EGFR-expressing metastatic colorectal cancer	ORR 23% combined with irinotecan	2004
Erlotinib	EGFR	Metastatic NSCLC with EGFR mutation	Improved PFS 5.2 to 10.4 mo	2013
		Maintenance treatment of NSCLC	Improved PFS HR 0.71	2010
		Unresectable pancreatic cancer	OS improved 6.0 to 6.4 mo	2005
		Refractory NSCLC	OS improved 4.7 to 6.7 mo	2004
Dasatinib	Multityosine kinase inhibitor	Chronic-phase CML, Philadelphia chromosome positive (Ph+)	Complete cytogenic response improved 66.2% to 76.8%	2010
		Refractory CML and ALL, Ph+	No phase III data	2006
Panitumumab	EGFR	EGFR-expressing metastatic colorectal cancer	PFS improved 60 to 96 d	2006
Sunitinib	Multikinase (VEGFR, PDGFR, KIT, FLT3, RET)	Pancreatic neuroendocrine tumor	PFS improved 5.4 to 10.2 mo	2011
		Renal cell carcinoma	ORR 25.5%-36.5%	2006
		GIST	TTP improved 6 to 27 wk	2006
Lapatinib	HER-2	ER/PR+, HER2+ breast cancer	PFS improved 13 to 35 wk	2010
		HER2+ breast cancer	TTP improved 18 to 24 wk	2007
Pazopanib	VEGFR	Advanced soft tissue sarcoma	PFS improved 1.6 to 4.6 mo	2012
		Advanced renal cell carcinoma	PFS improved 4.2 to 9.2 mo	2009
Vandetanib	VEGFR, EGFR	Medullary thyroid cancer	ORR improved 1% to 44%	2011
Crizotinib	c-Met, anaplastic lymphoma kinase (ALK)	ALK-positive NSCLC	PFS improved 3.0 to 7.7 mo	2011/2013
Axitinib	VEGFR	Renal cell carcinoma	Improved PFS 4.7 to 6.7 mo	2012
Bosutinib	Bcr-Abl, Src-family kinases	CML/ALL Ph+	No phase III data	2012
Cabozantinib	Pan-tyrosine kinase inhibitor	Metastatic medullary thyroid cancer	PFS improved 4.0 to 11.2 mo	2012
Ponatinib	Multikinase inhibitor	CML/ALL Ph+	Phase III trial stopped	2012
Regorafenib	Multikinase inhibitor	GIST	PFS improved 0.9 to 4.8 mo	2013
		Refractory metastatic colorectal cancer	Improved OS 5.0 to 6.4 mo	2012
Afatinib	EGFR, HER2, HER4	Metastatic NSCLC with mutant EGFR	PFS improved 6.9 to 11.1 mo	2013
Ibrutinib	Burton's tyrosine kinase	CML	ORR 58.3%	2014
		Mantle cell lymphoma	ORR 69%	2013
Ceritinib	ALK	ALK-positive metastatic NSCLC	ORR 54.6%	2014
Ramucirumab	VEGFR	Gastric cancer	OS improved 3.8 to 5.2 mo	2014
		Metastatic NSCLC	OS improved 9.1 to 10.6 mo	2014
Serine/threonine kinase inhibitors				
Vemurafenib	BRAFV600E	Melanoma V600E mutant	PFS improved 1.6 to 5.3 mo	2011
Trametinib	MEK1, MEK2	Melanoma BRAF V600E/V600K mutant	PFS improved 1.5 to 4.8 mo	2013
Dabrafenib	BRAF, CRAF	Melanoma BRAF V600E mutant	PFS improved 2.7 to 5.1 mo	2013
Other kinase inhibitors				
Sorafenib	Multikinase inhibitor (BRAF, VEGFR, PDGFR, FLT3, KIT)	Differentiated thyroid cancer	PFS improved 5.8 to 10.8 mo	2013
		Hepatocellular carcinoma	OS improved 7.9 to 10.7 mo	2007
		Renal cell carcinoma	PFS improved 84 to 167 d	2005
Temsirolimus	mTOR	Renal cell carcinoma	Improved PFS 3.1 to 5.5 mo	2007
Everolimus	mTOR	HER2-negative breast cancer	PFS improved 3.2 to 7.8 mo	2012
		Pancreatic neuroendocrine tumor	PFS improved 4.6 to 11.0 mo	2011
		Renal cell carcinoma	PFS improved 1.9 to 4.9 mo	2009
Idelalisib	Phosphoinositide-3 kinase	Relapsed CLL	PFS HR 0.18	2014
		SLL	ORR 58%	
		Follicular NHL	ORR 54%	

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TABLE 1 (*continued*)

Agent	Target	Disease Site	Clinical Results	Year FDA Approval
HDAC inhibitors				
Vorinostat	HDAC	Cutaneous T-cell lymphoma	ORR 30%	2006
Romidepsin	HDAC	Cutaneous T-cell lymphoma	ORR 34-35%	2009
Belinostat	HDAC	Refractory peripheral T-cell lymphoma	ORR 25.8%	2014
Panobinostat	HDAC	Refractory multiple myeloma	PFS improved 5.8 to 10.6 mo	2015
Proteasome inhibitors				
Bortezomib	Proteasome	Mantle cell lymphoma	PFS improved 14 to 25 mo	2014
		Multiple myeloma	ORR 28%	2003
Carfilzomib	Proteasome inhibitor	Multiple myeloma	ORR 23%	2012
PARP inhibitors				
Olaparib	PARP	Ovarian cancer with germline BRCA mutation	ORR 34%	2014

DOR: duration of response; ORR: overall response rate; PFS: progression-free survival; TTP: time to progression.
HR = Hazard Ratio DFS = Disease Free Survival OS = Overall Survival.

expressing a kinase-dead form of EGFR are viable with minimal defects [20]. Also, mice that express a kinase-inactive form of EGFR (V765G-EGFR) are fertile and show a significant reduction in intestinal polyps when crossed with APC^{Min} mice compared with APC^{Min} mice carrying wild-type EGFR. These findings suggest that EGFR kinase activity is important for tumorigenesis, but its physical presence is essential for cell survival. Along these lines, in patients with colorectal cancer, EGFR expression correlates with prognosis but not with response to EGFR inhibitors such as cetuximab. We recently reported our finding that degradation of EGFR is more efficacious than treatment with small molecule inhibitors and that this strategy can overcome resistance from an acquired EGFR mutation (T790M) [21]. We discuss this strategy in more detail later in this review.

Proteosome Inhibitors as Anticancer Agent

So far, the most successful drug targeting protein degradation is bortezomib (Velcade, Millennium Pharmaceuticals). It is approved for use in patients with multiple myeloma and non-Hodgkin's lymphoma. Bortezomib binds to the catalytic site of the 26S proteasome, ultimately inhibiting the degradation of proteins. Because the proteasome degrades most cellular proteins, this drug class has many side effects including peripheral neuropathy and myelosuppression [22]. A proposed mechanism of bortezomib's anticancer activity is the attenuation of IκB degradation which promotes inactivation of NF-κB. However, the true mechanism behind this drug's actions is poorly understood as countless proteins are affected by proteasome inhibition.

Given the lack of target specificity seen with proteasome inhibitors, more selective approaches that target the degradation of a specific oncoprotein or tumor suppressor are needed. One way to accomplish this goal is to target protein ubiquitination or neddylation processes. A novel inhibitor of neddylation, pevonedistat (MLN4924), is currently in clinical trials for hematologic malignancies and melanoma. Similarly, the E3 ubiquitin ligase MDM2 has been targeted in patients using the drug RO5045337A. Several recent reviews discuss these efforts in more detail. Below, we discuss novel approaches of targeting protein-protein interaction to target oncogenes and tumor suppressors.

Targeting Ubiquitination-Mediated Protein Degradation

Ubiquitination-mediated protein degradation is an exciting target for cancer therapy. The ubiquitination cascade consists of three enzyme groups: E1 is a group of ubiquitin-activating enzymes, E2 is a group of ubiquitin-conjugating enzymes, and E3 is a group of ubiquitin ligases. This cascade regulates the addition of ubiquitin moieties to specific proteins within a cell, leading to protein degradation. By altering these pathways, one can potentially manipulate the degradation of a protein. An example of this strategy is the use of 5-deazaflavin derivatives to inhibit the ubiquitin ligase MDM2, resulting in decreased degradation of p53, a tumor suppressor [23]. Although 5-deazaflavin derivatives inhibit MDM2-mediated p53 ubiquitination, they have poor substrate selectivity as they target a number of other kinases. An alternative strategy being studied is the use of Nutlins (particularly Nutlin-3) to disrupt the interaction between p53 and MDM2 [24]. Nutlins are currently in clinical trials for pediatric cancers containing wild-type p53.

Like p53, a reduction in p27 levels has been correlated with a poor prognosis in many types of cancer. Mechanistic studies indicate that the loss of p27 depends on SKP2 E3 ligase-mediated proteasomal degradation. A recent report describes a small molecule capable of disrupting a critical interaction between SKP2 with its partner SKP1 [25]. Similarly, the interaction of SKP2 with phosphorylated p27 requires the adapter protein CKS1 for degradation. Small molecule inhibitors capable of disrupting the SKP2:CKS1 interaction have been developed [26]. Another example of this strategy is targeting PML, a known tumor suppressor. PML is downregulated in many human malignancies because of enhanced ubiquitination-mediated proteasomal degradation. Agents that manipulate PML ubiquitination are in development.

Similar to the downregulation of tumor suppressors, the overexpression of oncoproteins is critical for cancer development and progression. The amount of a specific protein in a cell depends on the rate it is synthesized and its stability or half-life. So far, efforts to target an oncoprotein's stability have been limited. As discussed above, we believe that the physical presence of an oncoprotein, independent of its activity, can promote cancer progression. Therefore, inducing degradation of an oncogenic protein, as opposed to mere inhibition of its activity, is a promising strategy. A better understanding of the factors responsible for the stability of oncoproteins is needed to accomplish this.

A New Approach of Targeted Oncoprotein Degradation

EGFR Degradation: Disruption Of Homo- And Heterodimerization. We have studied the selective degradation of EGFR as a novel strategy to target EGFR-driven tumors. Under physiological conditions, EGFR is stable with a half-life greater than 12 hours. Its stability depends on the ability to form homo- or heterodimers as EGFR is highly unstable in its monomer state. When inactive, EGFR can form a dynamic heterocomplex with the chaperone protein HSP90, promoting stability [27]. Upon stimulation by

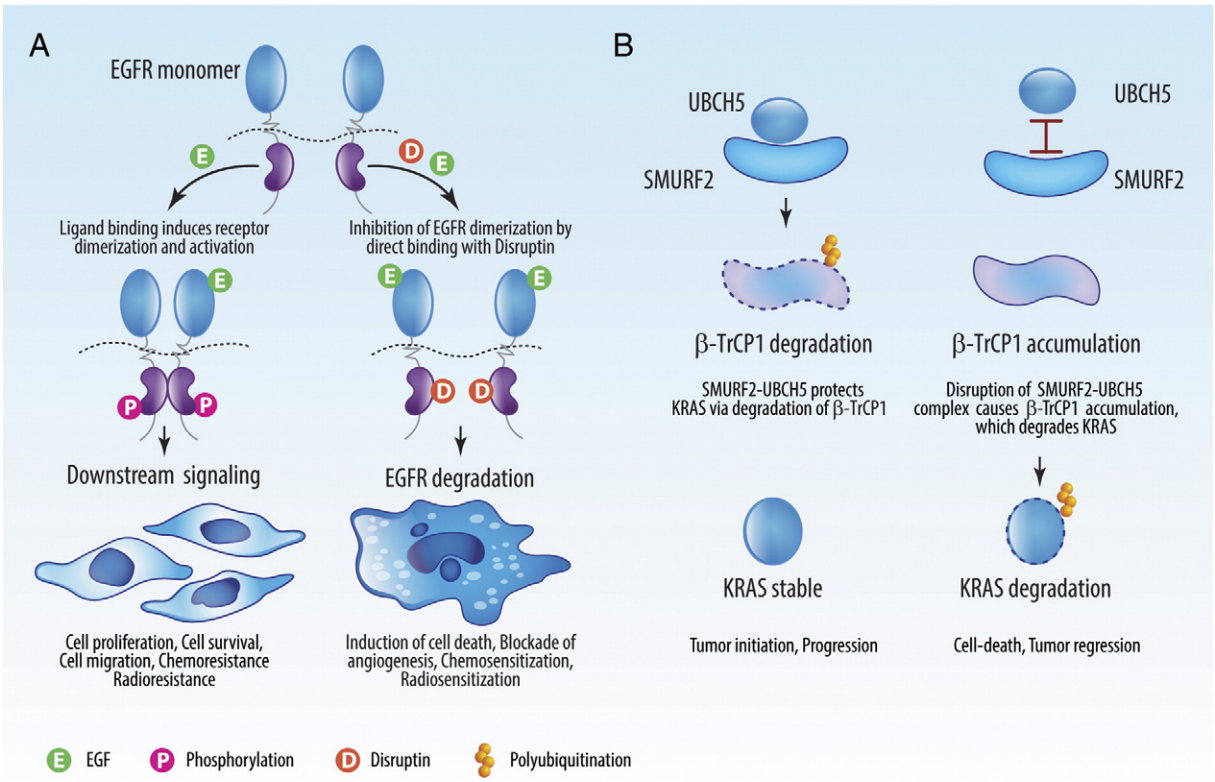

Figure 1. Disruption of protein-protein interactions to induce oncoproteins degradation. (A) Disruptin (D), a peptide containing an eight–amino acid stretch of human EGFR, is capable of disrupting the protein:protein interactions between EGFR:HSP90 and EGFR homodimers. These interactions are critical for maintaining EGFR stability. In the presence of high levels of EGF, as seen in the tumor microenvironment, treatment with Disruptin causes rapid degradation of EGFR. In preclinical models, treatment with Disruptin induces regression of EGFR-dependent tumors including those resistant to TKIs. (B) Interaction between the SMURF2:UBCH5 protein complex and β-TrCP1 plays a critical role in the maintenance of KRAS protein stability. The SMURF2:UBCH5 complex polyubiquitinates and degrades β-TrCP1, which indirectly protects KRAS from degradation. We found that the disruption of the SMURF2:UBCH5 complex can promote β-TrCP1 accumulation, leading to rapid KRAS degradation.

various growth factors, EGFR is phosphorylated and dissociates with HSP90 to form a homodimer with another EGFR molecule or a heterodimer with another EGFR family member (such as ErbB-2, -3, or -4) or related tyrosine kinase (such as c-Met). Based on these findings, we identified a critical eight–amino acid stretch (amino acids 768-SVDNPHVC-775) that lies within the αC-β4 loop region of the EGFR kinase domain corresponding to the binding region of HSP90 [28]. We found that mutating the first six amino acids in this sequence disrupts EGFR homo- and heterodimerization, making the protein highly unstable (half-life <3 hours). Using this knowledge, we developed a peptide named Disruptin (Figure 1*A*), which inhibits dimerization of EGFR, leading to rapid degradation. As cancer cells have higher levels of activated EGFR than normal tissue, Disruptin preferentially targets tumor cells expressing EGFR. Furthermore, Disruptin has therapeutic efficacy in preclinical xenograft models carrying the T790M mutation which makes tumors resistant to erlotinib. This demonstration of selective EGFR degradation with the peptide Disruptin represents a novel therapy for EGFR-driven tumors, including those that are TKI resistant [28].

EGFR Degradation: Targeting Receptor Trafficking. Receptor trafficking also plays a critical role in determining the fate of EGFR molecules. Monoubiquitination and polyubiquitination of EGFR are involved in its stability, localization, and functionality. In addition to its ability to signal from the cell membrane, EGFR is also involved in signal transduction after it is endocytosed. The endocytosed receptor can be recycled back to the cell membrane, or it can be trafficked to the multivesicular bodies for lysosomal degradation. Factors involved in EGFR trafficking can be categorized into two groups: 1) factors that promote receptor recycling and 2) factors that promote EGFR endocytosis and degradation. Among the trafficking factors that promote receptor stability, Vav proteins (guanine nucleotide exchange factors) have been shown to positively regulate EGFR signaling by attenuating receptor internalization and degradation. Similarly, SGEF and DYRK1A can protect EGFR from degradation by delaying lysosomal sorting. Factors like Odin/ANKS1A also interact with EGFR, increasing receptor recycling. In contrast, Ephrin A5 promotes EGFR degradation by cooperating with c-CBL, an E3 ligase known to polyubiquitinate this protein. Also, Presenilin, a component of gamma-secretase protease complex, causes EGFR degradation indirectly by negatively regulating the expression and activity of the RING-type ubiquitin ligase Fbw7. All of these molecules represent novel molecular targets for EGFR-driven tumors.

Because ubiquitination plays an important role in EGFR endocytosis, trafficking, and lysosomal degradation, various ubiquitin ligases and deubiquitinating enzymes have been shown to regulate EGFR stability. The most extensively studied E3 ubiquitin ligase responsible for EGF-mediated EGFR polyubiquitination is c-CBL.

In addition, AIP4/ITCH, pVHL, and UBE4B play roles in the polyubiquitination and degradation of EGFR [29,30]. We have recently identified that the HECT-type E3 ubiquitin ligase SMURF2 directly interacts with EGFR. SMURF2's ubiquitin ligase activity is critical for maintaining EGFR protein stability [31]. Similar to SMURF2, deubiquitinating enzymes such as USP18 and USP2A protect EGFR from degradation. USP18 negatively regulates the expression of a microRNA (miR-7) which blocks EGFR translation by binding to the 3'-UTR of the EGFR transcript. USP2A antagonizes EGFR endocytosis via deubiquitination. Knowledge of the factors responsible for maintaining EGFR protein stability will allow for the development of novel drugs targeting this important receptor.

Downregulation of Mutant KRAS Protein: siRNA

Mutant KRAS has a high prevalence in pancreatic (90% KRAS mutant), colorectal (50% mutant), and lung (30% mutant) cancers. Effective treatment strategies are greatly needed for patients with KRAS-driven tumors. Direct inhibition of this protein is not feasible because of high intracellular GTP concentrations (micromolar) compared with the picomolar affinity between KRAS and GTP. Because KRAS acts as an on/off switch for multiple signaling pathways, the predominant strategy for targeting mutant KRAS tumors has focused on inhibiting downstream pathways including RAF-MEK-ERK and PI3K-AKT-mTOR. These approaches have had limited success because of the activation of compensatory signaling. In contrast, various studies using mutant KRAS–specific siRNA-mediated knockdown have shown promise.

Early attempts using either an anti-KRAS ribozyme [32] or shRNA-mediated knockdown of KRAS [33] demonstrated tumor growth delay of mutant KRAS-driven NSCLC. However, in these studies, compensatory activation of STAT3 and EGFR signaling was noted, suggesting that targeting mutant KRAS alone may not be sufficient. Along this point, a recent report demonstrated better *in vivo* tumor control when mutant KRAS siRNA was combined with either RAF or PI3K siRNAs [34]. These studies confirm the proof-of-principle that siRNA-mediated physical ablation of mutant KRAS is an attractive therapeutic strategy; however, we believe that the clinical success of these therapies depends on the long-term and tumor-specific delivery of siRNA payloads, which remains a challenge. Recently, one such siRNA delivery system has been developed, called Local Drug Eluter (LODER). LODER is able to protect siRNA from degradation and promotes localized prolonged siRNA release, an essential requirement for clinical success [35]. Using this delivery system, mutant KRAS siRNA (siG12D LODER) was delivered in pancreatic tumor xenograft models successfully. Such a system is currently being tested in a phase I (NCT01188785) and a phase II (NCT01676259) clinical trial for unresectable locally advanced pancreatic cancer patients.

Downregulation of Mutant KRAS Protein: Targeting Ubiquitination Machinery

Targeting posttranslational modifications of KRAS is a promising alternative to siRNA-based methods. Supporting this idea is a recent report that identifies a small molecule capable of inhibiting KRAS through its interaction with prenyl-binding protein PDEδ, a step critical for KRAS localization to the endomembrane [36]. Our work related to this strategy has focused on targeting the ubiquitination machinery that regulates KRAS stability. Monoubiquitination of mutant KRAS is known to enhance its activity, amplifying downstream signaling [37]. Based on the mechanistic studies of other proteins, we speculated that monoubiquitination competes with β-TrCP1–mediated polyubiquitination to maintain RAS protein levels. Interestingly, we observed that, under physiological conditions, the GTP-bound active form of mutant KRAS has a similar half-life as the GDP-bound inactive form of wild-type KRAS. Our observation is in direct contrast with previous reports which show that an active protein has a shorter half-life than its inactive form. We hypothesized that this difference is due to enhanced monoubiquitination of the mutant form of KRAS. Based on our prior work, we hypothesized that the E3 ubiquitin ligase SMURF2 regulates the stability of GTP-bound mutant KRAS. As expected, we found that the loss of SMURF2 caused mutant KRAS to become highly unstable.

Although we discovered that SMURF2 ubiquitin ligase activity is critical in maintaining mutant KRAS protein stability, we found that SMURF2 does not monoubiquitinate mutant KRAS as hypothesized. Instead, it indirectly protects mutant KRAS by polyubiquitinating and degrading β-TrCP1 [38]. As a proof-of-principle study, we showed that si/sh-RNA targeting of SMURF2 attenuates the growth of mutant KRAS–driven tumors. Although the finding that SMURF2 silencing can degrade mutant KRAS and block the growth of mutant KRAS–dependent tumors is encouraging, direct inhibition of SMURF2 ubiquitin ligase activity is not a viable strategy because of the critical importance of SMURF2 in mitosis [39]. We further found that SMURF2 can monoubiquitinate its partner ubiquitin conjugating enzyme (E2) UBCH5 to form an E3:E2 complex required for β-TrCP1 degradation. These findings suggest that the SMURF2:UBCH5 complex is critical in maintaining mutant KRAS protein stability and could be explored to develop the novel anti-KRAS strategy proposed in Figure 1*B*.

Future Directions

Inhibition of oncogenic kinase activity has led to the discovery and development of novel therapies that benefit patients. However, kinase inhibition has significant limitations including the emergence of drug resistance leading to limited therapeutic durability. Targeting protein stability is an interesting alternative strategy that is potentially applicable to any oncoprotein or tumor suppressor. Strategies such as disrupting protein-protein interactions to destabilize an oncoprotein or to stabilize a tumor suppressor protein are in the early phases of development. Although targeting protein-protein interactions using synthetic agents is challenging, recent advancements in structural and computational proteomics allow us to identify novel synthetic mimetics capable of disrupting specific protein-protein interaction to induce oncoprotein degradation. Such an approach can abolish the presence of an oncoprotein, which, in theory, can overcome resistance to kinase inhibitors. Future studies will determine if this approach can improve the outcomes of patients.

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