RNA Interference in Cancer: Targeting the Anti-Apoptotic Protein c-FLIP for Drug Discovery

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Abstract: The silencing of genes by RNA interference (RNAi) has identified proteins involved in the resistance of cancers to chemotherapeutic drugs. Resistance is associated with defects in the apoptotic signaling pathways. In this article, we examine using RNAi to target the anti-apoptotic protein cellular FLICE-like inhibitory protein (c-FLIP) for drug development.

Key Words: RNA interference, c-FLIP, apoptosis, apoptotic pathways, drug discovery, cancer, caspases.

INTRODUCTION

Cancer accounts for ~23% of mortalities in the United States and is second only to heart disease in deaths each year [1]. Despite some therapeutic successes, the intrinsic or acquired resistance of cancers to chemotherapeutic drugs is a serious clinical problem and often leads to treatment failure. One of the challenges facing molecular oncology is the identification of these underlying drug resistance mechanisms, so that novel therapies can be developed to bypass these roadblocks. The RNAi-mediated silencing of genes from diverse signaling pathways has yielded key insights into the complexity of cancer and has identified specific signaling pathway components that cause drug resistance. Moreover, the resistance of malignant cells to drug therapy is often associated with defects in the apoptotic signaling pathways or expression of anti-apoptotic proteins such as the cellular FLICE-like inhibitory protein (c-FLIP) [2-4]. Furthermore, considerable amounts of c-FLIP are expressed in breast, ovarian, colon, glioblastoma, colorectal, and prostate cancers, and its expression is associated with chemotherapeutic drug and cytokine (TNF-α, Fas ligand, and TRAIL) resistance in a majority of these malignancies [5-8]. In this review, we will discuss the apoptotic signaling pathway components as RNAi targets, and report why we think that the anti-apoptotic protein c-FLIP is an excellent target for RNAi because of its key survival role in multiple cancer cell lines, as well as its function in tumorigenesis and tumor aggressiveness.

1. BIOLOGY OF RNAi

RNAi is a naturally occurring mechanism in eukaryotic cells that controls gene expression at the post-transcriptional level [9]. The regulation of endogenous genes by RNAi occurs *via* the production of short double-stranded RNA molecules termed siRNA *via* a multiprotein signaling pathway (for review, see [10-12]). For a brief review, long double-stranded RNA (dsRNA) precursors are introduced into the cytoplasm and processed by the RNase-III enzyme Dicer. Dicer cleaves the dsRNA into short 21-23 duplexes that are referred to as short interfering RNAs (siRNA), and these siRNA duplexes associate with the multiprotein RNA-inducing silencing complex (RISC), where the sense strand is degraded, leaving the antisense "guide" strand free to bind to the complimentary target mRNA. After the guide strand anneals to the target mRNA, the RISC residing enzyme Argonaute-2 (AGO2) cleaves and degrades the mRNA (Fig. **1**). Experimentally, the introduction of long dsRNA (>30 nts) into mammalian cells triggers the antiviral IFN response and shuts down the global expression of proteins [13, 14]. To limit this, RNAi can be accomplished by using small-interfering RNA (siRNA) duplexes that silence gene expression without inducing the interferon response. siRNAs can be directly introduced into cells by transfection or introduced *via* plasmids that express short-hairpin RNA (shRNA) precursors of siRNA, which are then processed to siRNAs by the enzyme Dicer (Fig. **1**).

2. APOPTOTIC SIGNALING PATHWAYS

Apoptosis is a normal physiological process that occurs during embryonic development and also maintains tissue homeostasis. Apoptotic cell death exhibits specific morphological and biochemical characteristics, including loss of plasma membrane asymmetry, chromatin condensation, genomic DNA fragmentation, cell membrane blebbing, and the formation of small vesicles from the cell surface termed apoptotic bodies [15]. Following apoptosis, the apoptotic bodies are engulfed by phagocytes, and thus an inflammatory response does not occur. The absence of functional apoptotic signaling can lead to many diseases including cancer [2, 16]. In fact, the absence of functional apoptotic signaling or the expression of anti-apoptotic proteins, such as c-FLIP, has been linked to enhanced tumorigenesis and poor clinical response to chemotherapeutic drugs [17-22].

The two well-studied apoptotic signaling pathways are the cell surface death receptor pathway and the mitochon-

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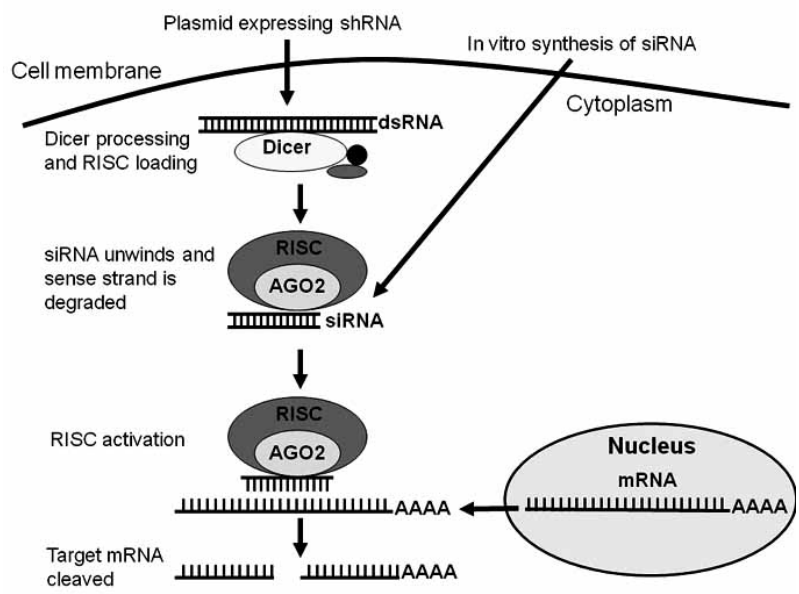

Fig. (1). Mechanism of RNAi. Short-hairpin RNAs (shRNAs) that are encoded by plasmids are processed by the Dicer enzyme complex to produce siRNA. siRNA can also be synthesized *in vitro* and complexed with other molecules for efficient delivery into the target cells. The siRNA is loaded into the RNAi-induced silencing complex (RISC), where the sense strand is degraded, leaving the antisense "guide" strand free to hybridize with its complementary sequence in the mRNA. Once bound, the mRNA is degraded by Argonaute 2 (AGO2), which resides in the RISC.

drial pathway (Fig. **2**). Activation of these two pathways triggers the activation of caspases, a family of cysteine proteases that degrade a critical set of cellular proteins leading to the autodestruction of the cell. Caspases normally exist as inactive precursors in the cell, called procaspases, which are cleaved to produce the active enzymes during apoptosis. Furthermore, caspases involved in apoptosis are categorized as initiator or effector caspases, depending on their structure and function [23, 24]. In the death receptor pathway, apoptotic signaling is initiated when death receptors at the cell surface bind to their cognate death ligands triggering a conformational change that is transmitted through the cell membrane. The three most studied death receptor/ligand pairs are members of the TNF receptor (TNFR) superfamily, which have roles in proliferation, differentiation, and apoptosis. The death receptors include TNF-α receptor, Fas (APO-1/CD95), and TRAIL receptors DR4 (also known as TRAIL receptor 1), and DR5 (also known as TRAIL receptor 2), which bind to their cognate ligands TNF-α, Fas ligand, and TRAIL, respectively. Additional TNFR superfamily members inhibit apoptosis by serving as cell surface decoy receptors that compete with functional receptors for death ligand binding [4, 25]. The death receptors contain a conserved intracellular death domain (DD) of about 80 amino acids, which upon ligand binding interacts with the adaptor proteins FADD or TRADD [26]. FADD then recruits the initiating caspases-8 and -10 through homophilic death effector domain (DED) interactions to form the death inducing signaling complex (DISC) [27, 28]. The close proximity of the initiator caspases in the DISC facilitates their dimerization and activation, leading to the activation of downstream effector caspases including caspase-3 and the cleavage of the pro-apoptotic protein Bid [29]. Cleaved Bid activates downstream pro-apoptotic proteins such as Bax and Bak which promote cytochrome *c* release from the mitochondria into the cytosol, thereby linking the death receptor and mitochondrial pathways [30]. The cytosolic cytochrome *c* associates with caspase-9, dATP, and APAF-1 forming the apoptosome complex, leading to the activation of caspase-9. Active caspases-8 and -9 lead to the activation of caspase-3, which triggers the cleavage of many cellular proteins such as protein kinase C, poly(ADP-ribose) polymerase, gelsolin, DNA fragmentation factor-45 (DFF45), and fodrin inducing apoptosis [31-33].

3. INHIBITORS OF APOPTOSIS

Apoptotic signaling *via* the death receptor and mitochondrial pathways is regulated in part by the inhibitor of apoptosis (IAP) proteins (XIAP, survivin, c-IAP-1, and cIAP-2) that directly inhibit caspases (for a review, see reference [34]), and by Bcl-2 family members [35, 36]. The Bcl-2 family of proteins consists of pro-apoptotic members (Bid, Bak, Bad, or Bax) that induce mitochondrial outer membrane permeabilization, which triggers the release of cytochrome *c* and caspase-activating proteins, while the anti-apoptotic members (Bcl-2, Mcl-1, or Bcl-X_L) maintain the integrity of the mitochondria by opposing the pro-apoptotic effects of Bax and Bak. Besides the IAP and Bcl-2 family of proteins, the c-FLIP isoforms are important regulators of death receptor-induced apoptosis because of their ability to bind to

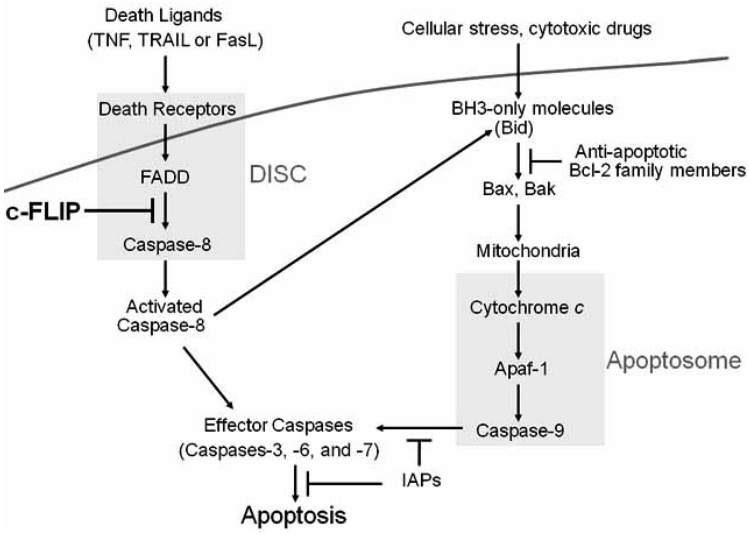

Fig. (2). Schematic diagram of apoptotic signaling pathways. The death receptor pathway is activated by the binding of the death ligands FasL, TRAIL, or TNFα to their cognate receptors, which triggers receptor aggregation leading to the recruitment of the adaptor molecule FADD and caspase-8, thereby forming the death inducible signaling complex (DISC). Caspase-8 becomes activated upon recruitment and initiates apoptosis by directly activating downstream effector caspases. The mitochondrial pathway is initiated within the cell due to intracellular changes, such as DNA damage, resulting in the activation of the pro-apoptotic Bcl-2-family members Bid, Bax, and Bak, which trigger the permeabilization of the mitochondrial outer membrane culminating in the release of cytochrome *c* into the cytosol. The cytosolic cytochrome *c* associates with caspase-9, dATP, and Apaf-1 to form the apoptosome complex, leading to the activation of caspase-9 and downstream effector caspases. The death receptor and mitochondrial pathways can be interconnected by the caspase-8-dependent cleavage of Bid. Activation of caspases is negatively regulated at the receptor level by c-FLIP, which prevents caspase-8 activation, and at the mitochondria by anti-apoptotic Bcl-2 family members. IAPs inhibit caspase activation downstream of both pathways by directly inhibiting the activation of caspase-9 and effector caspases. The DISC and apoptosome are highlighted in grey.

caspase-8, caspase-10, FADD, and DR5, thereby preventing DISC formation [3]. Furthermore, the RNAi-based knockdown of the Bcl-2, Bcl-X_L, XIAP, survivin, and c-FLIP genes has been shown to sensitize diverse cancers to chemotherapeutic drugs, FasL, and TRAIL *in vitro* and regress tumors *in vivo* (Table **1**). In the following sections, we will begin by describing the function and characteristics of c-FLIP, and then discuss our findings as well as studies by other laboratories showing that the RNAi-mediated knockdown of c-FLIP triggers spontaneous apoptosis in diverse cancer cell types, and address the rationale for using c-FLIP as an anticancer therapeutic target.

3.1. c-FLIP

c-FLIP was originally identified in viruses by screening databases using a generalized profile constructed from DEDs from FADD, FLICE (caspase-8), and Mch4 (caspase-10) [37]. Based on the characterization of viral c-FLIPs, the mammalian homolog was found and called c-FLIP [38]. To date, 11 distinct c-FLIP splice variants have been identified, and three are expressed as proteins: the 55 kDa long form (c-$FLIP_L$), 26 kDa short form (c-$FLIP_S$), and a 24 kDa form (c-$FLIP_R$) [38, 39]. All c-FLIP isoforms contain two N-terminal DED domains, but only c-$FLIP_L$ contains a C-terminal caspase domain with structural similarity to caspase-8. In the caspase-like domain of c-$FLIP_L$, the catalytically active cysteine is replaced by tyrosine, rendering the molecule proteolytically inactive [40].

3.2. The Function of c-FLIP

The c-FLIP proteins are recruited to the DISC by their DED domains and compete with the DED domain-containing initiating caspases for FADD binding sites [4]. c-$FLIP_S$ and c-$FLIP_R$ both inhibit death receptor signaling by inhibiting caspase-8 activation [39, 41]. c-$FLIP_L$ has a dual function at the DISC because it can either function as a pro-apoptotic factor by directly interacting with caspase-8, or it can act as an anti-apoptotic factor like c-$FLIP_S$ by inhibiting caspase-8 activity as well as activating cytoprotective pathways (see below) [22, 42]. The pro-apoptotic function of c-$FLIP_L$ is supported by studies showing that c-FLIP knockout mice die early in embryonic development due to heart failure, a defect also observed in FADD- and caspase-8-knockout mice [43, 44]. However, the majority of studies using many different cancer cell types have shown that the role of c-$FLIP_L$ is generally anti-apoptotic. For instance, treating cancer cells with Taxol, doxorubicin, or cisplatin induces the downregulation of c-FLIP and sensitizes the cells to these chemotherapeutic agents [45-47]. We recently showed that the knockdown of c-FLIP using a c-FLIP-specific siRNA triggered spontaneous apoptosis in MCF-7 breast cancer cells [48] (Table **2**). Besides our results, other studies have

Table 1. RNAi Targeting of Apoptotic Pathway Components for Cancer Therapy

Target	Cancer Type	Result	Reference
c-FLIP			
	Breast	Apoptosis	[72]
	Colorectal	Apoptosis	[73]
	Colorectal	Growth inhibition	[65]
	Lung	Apoptosis	[22]
	Lymphoma	Apoptosis	[74]
	Pancreatic	Apoptosis	[75]
	Breast	Apoptosis	[76]
Bcl-2			
	Prostate	Apotosis	[77]
	Lung	Apoptosis	[78]
	Cervical	Apoptosis	[79]
	Gastric	Growth inhibition	[80]
	Breast	Apoptosis	[81]
Bcl-XL			
	Liver	Apoptosis	[82]
	Colon	Apoptosis	[83]
Survivin			
	Breast	Apoptosis	[84]
	Breast	Apoptosis	[85]
	Bladder	Growth inhibition	[86]
XIAP			
	Breast	Apoptosis	[81]
	Renal	Apoptosis	[87]
	Glioma	Apoptosis	[88]

shown that the silencing of c-FLIP using c-FLIP-specific siRNA induces apoptosis in colorectal, lung, and prostate cancer cell lines (Table **2**). However, we were the first to clearly define the mechanism by which the silencing of c-FLIP induces spontaneous apoptosis. We showed that c-$FLIP_L$, DR5, FADD, and caspase-8, but not c-$FLIP_S$ or DR4, exist in a pre-assembled apoptotic inhibitory complex (AIC) in MCF-7 cells. The absence of c-$FLIP_L$ from this complex triggers spontaneous FADD- and DR5-dependent caspase-8 activation, leading to the cleavage of Bid to tBid. The production of tBid leads to mitochondrial disintegration, cytochrome *c* release, and activation of caspases-9, -6 and -7. Interestingly, c-FLIP siRNA-mediated apoptosis is blocked by inhibiting caspase-8 or -9 using the caspase-specific inhibitors z-IETD-fmk or z-LEHD-fmk, respectively, indicating that both the death receptor (caspase-8) and mitochondrial (caspase-9) apoptotic pathways are required for transducing death signals following c-FLIP knockdown. Moreover, the knockdown of c-$FLIP_S$ expression only had a minor effect on spontaneous apoptosis, indicating that the presence of c-$FLIP_L$ in the AIC plays the major role in preventing spontaneous apoptosis in MCF-7 cells.

Our findings also showed that both c-FLIP isoforms promote the proliferation of MCF-7 cells, but c-$FLIP_L$ appears to be more important than c-$FLIP_S$ in establishing long term survival [48]. These findings are agreement with other studies showing that c-FLIP is a cytoprotective protein involved in the activation of survival pathways in multiple cell types. For instance, the caspase-8-dependent cleavage of c-FLIP pro-duces a N-terminal p22 fragment that directly activates NFκB, leading to the proliferation of lymphocytic cells [49], and the overexpression of c-$FLIP_L$ has been

Table 2. c-FLIP Knockdown Induces Spontaneous Apoptosis in Different Cancer Cell Lines

Cell Line/Treatment	Percent Apoptosis	Reference
[a]MCF-7/control siRNA MCF-7/c-FLIP siRNA	5 45	[48]
[b]A549/control siRNA A549/c-FLIP siRNA	0 33-35	[22]
[c]HCT116/control siRNA HCT116/c-FLIP siRNA	15 45	[65]
[d]LNCaP/control siRNA LNCaP/c-FLIP siRNA	10 30	[66]
[d]PC3/control siRNA PC3/c-FLIP siRNA	5 30	[66]

[a]MCF-7 breast cancer cells were transfected with 100 nM non-targeting siRNA or c-FLIP-specific siRNA for 48 h, and apoptosis was determined by staining the cells with annexin V and propidium iodide followed by flow cytometry analysis.

[b]A549 lung cancer cells were transfected with 80-120 nM of non-targeting siRNA or c-FLIP-specific siRNA for 48 h, and apoptosis was determined by measuring the percentage of subdiploid cells by flow cytometry.

[c]HCT116 colorectal cancer cells were transfected with 10 nM of non-targeting siRNA or c-FLIP-specific siRNA for 48 h, and apoptosis was determined by measuring the percentage of subdiploid cells by flow cytometry.

[d]LNCaP or PC3 prostate cancer cells were transfected with 10 nM of non-targeting siRNA or c-FLIP-specific siRNA for 48 h, and apoptosis was determined by measuring the percentage of subdiploid cells by flow cytometry.

demonstrated to activate NFκB and promote survival signaling [50, 51]. Furthermore, c-FLIP$_L$ was shown to interact with proteins such as TRAF1, TRAF2, Raf-1, and RIP, which are known components of the TNFR-mediated NFκB activation pathway [52]. Recently, it has been demonstrated that c-FLIP$_L$ promotes the motility of cancer cells by activating ERK and FAK signaling [53]. Additionally, the overexpression of c-FLIP$_L$ blocked the proteosomal degradation of β-catenin, and the elevated β-catenin levels were shown to induce either Wnt signaling or cyclin D expression, and thereby enhance the proliferation and cell cycle progression of cancer cells [54, 55]. Studies have also shown that c-FLIP overexpression can promote carcinogenesis and aggressiveness of endo-metrial and cervical cancers [56, 57]. These studies delineate the multifunctional role of c-FLIP in diverse signaling pathways that regulate apoptosis, proliferation, carcino-genesis, and the survival of cancer cells.

4. RNAi-TARGETING OF c-FLIP FOR CANCER THERAPY

Therapeutic modalities that lower the threshold of cancer cell apoptosis should lead to more effective treatment of cancers. For example, strategies to inhibit the expression of anti-apoptotic proteins such as c-FLIP not only trigger spontaneous apoptosis in certain cancer types, but also sensitize cancer cells to chemotherapeutic agents, potentially allowing lower doses to be administered to the patient and decreasing drug-induced systemic toxicities. For example, we have shown that CCRF-HSB-2 human lymphoblastic leukemia cells transfected with an antisense c-FLIP plasmid abrogated c-FLIP$_S$ and c-FLIP$_L$ expression and triggered a 20% increase in Taxol-induced apoptosis [45]. These findings reveal that the c-FLIP isoforms are key factors in making cancer cells resistant to the apoptotic effects of Taxol.

Therefore, the development of RNAi-based therapeutics to target the c-FLIP gene *in vivo* may revolutionize the way cancers are treated by directly inducing cancer cell apoptosis or by sensitizing cancers to chemotherapeutic agents. However, challenges in siRNA design, delivery, and stability must be overcome to allow for RNAi-based drugs to move from the bench to the clinic.

5. EFFECTIVE DESIGN, DELIVERY, AND STABILITY OF siRNA

For RNAi-based gene silencing to work, the duplex siRNA has to be designed effectively to target homologous sites within the target mRNA and to minimize off-target effects due to similarities in nucleic acid sequences [11, 58]. By using the correct software and sequence analysis algorithms, a specific siRNA target sequence for a particular gene can be identified which will minimize the off-target effects [59]; for a list of companies selling RNAi-related products, see reference [14]. The off-target effects can arise if partial sequence homology occurs in the 3′UTR region of a target mRNA and an off-target mRNA [60-62]. Moreover, siRNA duplexes containing certain GU-rich sequences such as 5′-UGUGU-3′ or 5′-GUCCUUCAA-3′ may trigger TNF-α, IL-6, and IFN-α activation by directly interacting with Toll-like receptors (TLRs) [63, 64]. Effective siRNA design strategy must account for possible off-target effects and each siRNA must be empirically tested in a particular target system to identify selective and potent siRNA drug candidates.

One of the main limitations for translating RNAi technology from an effective research tool to a feasible therapeutic strategy is the efficient delivery of siRNAs to the targeted cell type *in vivo*. The RNAi used to silence cancer

genes can be derived from DNA vectors, viral vectors, or naked siRNA. Because of naked siRNA's molecular weight and polyanionic nature, it does not freely cross the cell membrane and must be complexed with a delivery agent such as liposomes or lipocomplexes, or conjugated with peptides, polymers, or antibodies [11, 13]. Lipocomplexes are formed by mixing the siRNA with a commercially available transfection reagent such as Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, CA). Lipocomplexes have been extensively used to deliver siRNA *in vitro* and *in vivo*. For instance, we have used lipocomplexes of c-FLIP siRNA to successfully knockdown the c-FLIP gene and induce spontaneous apoptosis in MCF-7 breast cancer cells *in vitro* [48] (see Table **2**), and *in vivo* by directly injecting the c-FLIP siRNA lipocomplexes into MCF-7 mouse xenografts (Day *et al.*, unpublished data). Lipocomplexes of c-FLIP siRNA have also been used to successfully silence the c-FLIP gene and trigger spontaneous apoptosis in A549 lung cancer cells [22], HCT116 colorectal cancer cells [65], and in LNCaP and PC3 prostate cancer cells [66] (see Table **2**). Furthermore, c-FLIP siRNA lipocomplexes were injected into HCT116 colorectal tumor xenografts and decreased tumor growth [65]. These studies show that c-FLIP siRNA lipocomplex formulations can be used to successfully knockdown the c-FLIP gene in various cancer cell types.

Exogenously administered siRNA should accumulate at the site of target organs or tissues at sufficient levels to exert its biological action with minimal effects on adjacent tissues. Unmodified or naked siRNAs are not stable in blood and serum, and have short half-lives *in vivo* because of degradation by nucleases. Chemical modifications to the backbone, base, or ribose of the siRNA can increase its half-life and enhance the biological activity of the molecule without affecting its gene knockdown activity [12, 67]. Specifically, modifying the 2′ position of the ribose by a 2′-O-methylpurine or 2′-fluoropyrimidine increases the stability of the siRNA by providing resistance to endonucleases [68-70]. For example, 2′-O-methylpurine modified siRNAs have been shown to provide greater protection against *in vivo* hepatitis B virus infection compared to the unmodified siRNAs [71]. More work still needs to be done to produce siRNAs that can be delivered to target tissues with sufficient stability to silence target gene expression and achieve beneficial therapeutic outcomes.

CONCLUDING REMARKS

The identification of specific genes involved in oncogenesis has greatly been expedited by using RNAi-based strategies. We believe that the identification of these genes will lead to the development of new therapeutic agents to combat cancer. We envision that RNAi-targeted therapies towards key regulatory genes such as c-FLIP will prove useful as monotherapy to treat certain types of cancers, as well as being used in conjunction with chemotherapy, cytokine therapy, or radiotherapy to sensitize resistant types of cancers. However, the process of translating the promising *in vitro* and preclinical animal model results to the more complex clinical arena will be a challenge. Progress in RNAi-based research that overcomes the hurdles of effective delivery, stability, and off-target interference will allow for this powerful tool to be used to treat diverse malignancies in the clinic.

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